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A Bacteriolytic Principle Associated with Cultures of *Bacillus cereus*

By J. R. NORRIS

Department of Agriculture, University of Leeds

SUMMARY: A lytic principle associated with cultures of *Bacillus cereus* is described and some of its properties discussed. The principle is relatively non-specific, acting on several species of aerobic spore-formers. It has properties in common with similar agents reported recently and comparisons with these are made. Lysis results in the loss of cell contents whilst at least part of the cell-wall structure remains intact.

Delpy & Chamsy (1949) found that in suspensions of old cultures of *Bacillus anthracis*, incubated in the presence of 1/20,000 thiomersalate, the vegetative rods underwent lysis which was complete in 4 days, the spores retaining their viability and serological properties. It is an extension of this observation that has led to the demonstration of a bacteriolytic principle associated with cultures of *B. cereus*.

METHODS

Organisms. The strain which produces the lytic principle used in this work (*Bacillus cereus* M. 8) was originally isolated from Egyptian soil (Mahmoud, 1955). It satisfied the main diagnostic criteria of Smith, Gordon & Clark (1952) and was strongly lecithinase-positive. It has been maintained by subculture on nutrient agar at 30°. The other organisms used for testing the principle were *B. subtilis* NCTC 3610 and the following strains from the Wellcome Research Laboratories: *B. circulans* 2925, *B. sphaericus* 1652, *B. polymyxa* 2002, *B. alvei* 2198 and *B. brevis* 2934 (Knight & Proom, 1950).

Thiomersalate. The preparation used was a 1/1000 solution of sodium mercurithiosalicylate obtained from Eli Lilly and Co. Ltd., Basingstoke.

Filter membranes. Courtauld's cellulose acetate membranes were used.

Culture media. Cultures were grown on Oxoid nutrient agar in 20 oz. Roux bottles which were each inoculated with 1 ml. of an overnight broth culture and incubated at 30°. For fluid cultures Oxoid nutrient broth was inoculated with a loopful of an overnight broth culture and incubated at 30°, with gentle shaking, in a water bath.

The production of the lytic principle. A Roux bottle containing nutrient agar was inoculated with *Bacillus cereus* M 8 and incubated for 12 hr. at 30°. The growth was then washed off into 10 ml. of sterile distilled water and the organisms centrifuged down to give an actively lytic supernatant fluid which could then be treated with thiomersalate or filtered through a sterilizing membrane as required.

The production of a bacterial suspension for use as substrate in the assay of lytic activity. For assaying lytic activity it was essential to use an organism

which showed no tendency to autolyse when suspended in distilled water (with or without thiomersalate) or in a preparation of the lytic principle which had been inactivated by heat. Most of the species investigated did in fact autolyse to some extent under these conditions, but *Bacillus sphaericus* 1652, whilst fully susceptible to the lytic principle, was stable in its absence and this organism was used throughout.

Bacillus sphaericus 1652 was grown in nutrient broth for 12–16 hr., centrifuged down, washed twice in sterile distilled water and resuspended in sterile distilled water to give a concentration of approximately 4×10^8 organisms/ml. This concentration, when diluted 10 times, gave a reading of about 100 units on the EEL nephelometer with the instrument set at two-thirds maximum sensitivity.

Method of assay of lytic activity. Samples (8.5 ml.) of the preparations to be tested were placed in sterile rubber-stoppered test tubes; 1 ml. of the *Bacillus sphaericus* 1652 suspension and 0.5 ml. of a 1/1000 solution of thiomersalate were then added to give a final thiomersalate concentration of 1/20,000. Each assay was performed in duplicate, the tubes being incubated in a water bath at 37° and nephelometer readings taken at intervals.

During the lysis of initially viable organisms there was a preliminary increase in nephelometer reading. The extent of this increase tended to differ from test to test and interfered with the comparison of the influence of variables such as temperature on the rate of lysis. When the *B. sphaericus* suspension was allowed to incubate in the presence of 1/20,000 thiomersalate for 3 hr. before addition of the lytic agent, no initial increase of optical density took place and a suspension pretreated in this manner was used whenever direct comparisons were to be made. It should be emphasized that such organisms were non-viable, since when washed *B. sphaericus* 1652 was suspended in distilled water there was a rapid decrease in viable count, the rate of which was not affected by the addition of thiomersalate (Fig. 1).

Staining techniques. The Gram method was carried out in the usual way with an acetone/ethanol mixture as decolorizing agent (Kopeloff & Cohen, 1928). Robinow's tannic acid-crystal violet method (Robinow, 1945) was used to stain cell walls.

Viable counts. In the absence of thiomersalate viable counts were performed in the usual way, using quarter-strength Ringer's solution as diluent and nutrient agar as the medium. When thiomersalate was present it was inactivated by the incorporation of glutathione to 3×10^{-3} M into the medium.

RESULTS

Lytic activity and age of culture

Preliminary experiments showed that the lytic activity associated with fluid cultures of *Bacillus cereus* M 8 was of a low order compared with that of cultures grown on solid media. A number of nutrient agar cultures was set up in Roux bottles and incubated at 30°. The total growth from a bottle was harvested into 10 ml. sterile distilled water at 8, 12 and 16 hr., the organisms

centrifuged down and the supernatant fluid treated with thiomersalate and assayed for lytic activity against *B. sphaericus* 1652. Comparison of the initial rates of decrease in nephelometer reading indicated that there was little difference in the activities of the three preparations (Fig. 2). A 12 hr. incubation period was adopted for the preparation of the lytic principle.

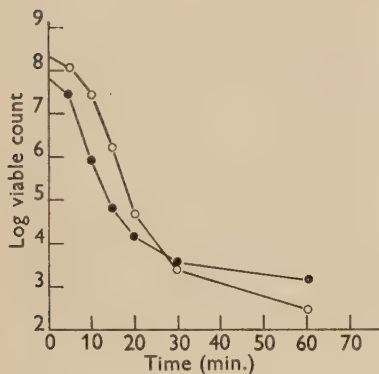


Fig. 1

Fig. 1. The loss of viability of washed vegetative organisms from a 16 hr. broth culture of *Bacillus sphaericus* when suspended in distilled water. In the presence ○—○ and absence ●—● of 1/20,000 thiomersalate.

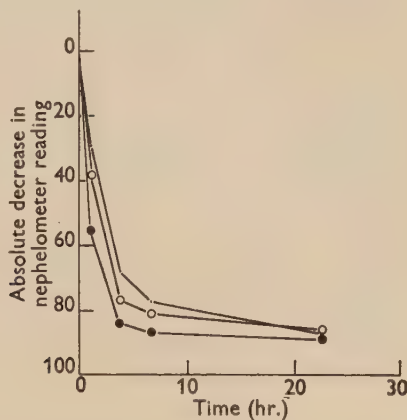


Fig. 2

Fig. 2. A comparison of the rates of lysis of vegetative organisms of *Bacillus sphaericus* suspended in supernatant fluids derived from cultures of *B. cereus* in the presence of thiomersalate. *B. cereus* cultures harvested at —○— 8 hr., ●—● 12 hr. and ○—○ 16 hr.

Since Ivanovics & Alföldi (1955) found that their agent, Megacine, was released during the lysis of the organism which produced it, the deposit from the 12 hr. culture of *Bacillus cereus* M8 in the above experiment was washed and resuspended in sterile distilled water to which thiomersalate was added. Autolysis, as judged by nephelometer reading and microscopically, occurred at 37° over a period of 24 hr. and samples of supernatant fluid taken at intervals were assayed for lytic activity against *B. sphaericus* 1652. There was no significant increase in lytic activity accompanying autolysis.

Properties of the lytic principle

Filterability. There was only slight loss of activity on passage through a membrane filter.

Optimum temperature for activity. Replicate assays were set up in water baths at different temperatures and the rates of lysis compared (Fig. 3); activity was optimal at 37°. Controls consisting of suspensions of *Bacillus sphaericus* 1652 in heat-inactivated preparations of the lytic principle, and similar suspensions in distilled water, both containing 1/20,000 thiomersalate, were set up at all the different temperatures and showed no significant change in optical density during the course of the experiment.

Dialysability. A preparation was dialysed in a cellophan bag against running water at 4°. There was no significant loss of activity at 20 hr. and the agent appeared to be non-dialysable.

Thermal stability. Samples of an active preparation were heated for 30 min. at different temperatures in water baths and their activities compared (Fig. 4). Loss of activity was almost complete following exposure to 70° for 30 min., and complete at 80° for the same length of time.

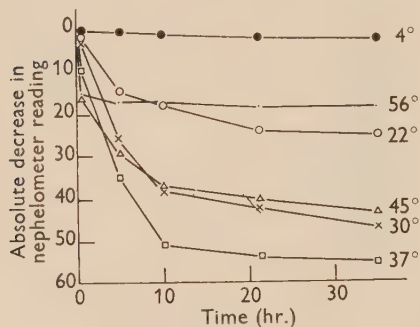


Fig. 3

Fig. 3. The effect of temperature on the rate of lysis of vegetative *Bacillus sphaericus* suspended in a preparation of the *B. cereus* lytic principle + 1/20,000 thiomersalate.

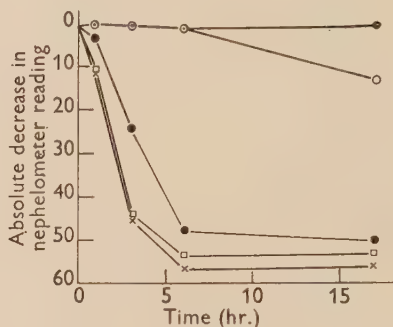


Fig. 4

Fig. 4. The thermal stability of the *Bacillus cereus* lytic principle. Vegetative *B. sphaericus* was suspended in preparations of the principle in the presence of thiomersalate. Preparation unheated \square — \square , and heated for 30 min. at \times — \times 55°, \bullet — \bullet 63°, \circ — \circ 70° and \bullet — \bullet 80°.

Optimum pH value. The normal pH value of the preparation was pH 8.0–8.2 and adjustment of reaction to the acid side resulted in considerable precipitation of material which interfered with the use of the nephelometer so that it was not possible to survey the activity at a range of pH values.

Specificity. The agent was tested against strains of the following members of the genus *Bacillus* and lysed all of them: *B. cereus*, *B. circulans*, *B. polymyxa*, *B. subtilis*, *B. alvei*, *B. brevis*, *B. sphaericus*. There was no lysis of *Staphylococcus aureus* (Oxford strain) or a strain of *Escherichia coli*.

Lecithinase and gelatinase activities. Complex preparations derived from a strongly proteolytic organism such as *Bacillus cereus*, may be expected to possess a variety of enzymic properties, and tests for lecithinase and gelatinase activities were both strongly positive. What part, if any, such activities play in the lytic process remains to be shown.

Action on heat-killed organisms. Vegetative *Bacillus sphaericus* 1652, killed by heating at 80° for 20 min., was rapidly lysed by preparations of the principle. Lysis of such organisms was not preceded by an increase in turbidity of the suspension as was noted earlier with unheated organisms (Fig. 5).

Cytological changes during lysis. Examined by the Gram method, lysis of *Bacillus sphaericus* 1652 appeared to consist of two distinct stages. The initial decrease in turbidity was associated with a loss of Gram-positive reaction. The rods, initially uniformly Gram-positive, rapidly became granular and the

Gram-positive granules then became fewer in number and stained less intensely. At the end of this phase the organisms stained well with the counterstain. During the second phase staining became fainter until the organisms appeared as barely visible ghosts; finally only debris was seen. When such a lysed preparation was stained with tannic acid-crystal violet, however, the cell walls were seen to be intact although almost devoid of contents, an observation confirmed by electron microscopy (Pl. 2, fig. 6). The details of lysis were essentially similar with all the seven organisms studied. Control suspensions of vegetative organisms in heat-inactivated lytic principle showed none of these changes during the course of the experiment (Pl. 2, fig. 5).

Bactericidal activity. The bactericidal activity of sterile (membrane filtered) thiomersalate-free preparations of the principle was investigated by adding 1 ml. of a 16 hr. broth culture of test organism to 9 ml. of the lytic preparation and following changes in viable count over a period of 15 hr. Controls consisted of similar inocula added to heat-inactivated material. The preparations showed a marked bactericidal activity against *Bacillus cereus*, *B. subtilis* and *Staphylococcus aureus* but did not affect *Bacillus sphaericus*, *B. circulans* or *Escherichia coli*. Death did not occur in the controls, and in most cases active growth took place (Table 1).

Table 1. *The bactericidal activity of the lytic principle from Bacillus cereus M 8*

1 ml. of an overnight broth culture of each test organism was added to 9 ml. of filtered lytic principle preparation, and the changes in viable count followed during incubation at 37°.

Test organisms	Organisms suspended in solution containing lytic principle			Organisms suspended in solution containing lytic principle inactivated by heating at 80° for 20 min.		
	Time of observation (hr.)			Time of observation (hr.)		
	0	5	16	0	5	16
	Viable counts (organisms/ml.)					
<i>Bacillus cereus</i>	235×10^3	24×10^3	162×10^1	219×10^3	51×10^4	30×10^5
<i>B. subtilis</i>	42×10^4	16×10^4	89×10^2	38×10^4	42×10^4	95×10^5
<i>Staphylococcus aureus</i>	71×10^4	9×10^1	18×10^0	77×10^4	23×10^4	99×10^4
<i>B. sphaericus</i>	140×10^4	26×10^5	162×10^6	131×10^4	22×10^5	22×10^6
<i>B. circulans</i>	52×10^4	127×10^3	187×10^4	46×10^4	59×10^4	48×10^6
<i>Escherichia coli</i>	57×10^5	65×10^5	46×10^6	52×10^5	74×10^5	57×10^6

The role of thiomersalate in the lytic process. Reference to Figs. 4–6 shows that in each of the control preparations vegetative *Bacillus sphaericus* 1652 showed no tendency to lyse in the absence of active lytic principle, and it may be concluded that thiomersalate had no lytic activity of its own under the conditions described. Thiomersalate proved to be essential for the initial increase in turbidity of the suspensions of *B. sphaericus* 1652 which were undergoing lysis, and its presence greatly facilitated the action of the lytic principle (Fig. 6).

DISCUSSION

Early reports of bacteriolytic activity associated with spore-forming aerobic bacteria are numerous (Waksman, 1945). Recently attention has been redirected to this subject by workers who have paid more attention to characterizing the agents concerned. Ivanovics & Alföldi (1955) induced rapid lysis in cultures of certain strains of *Bacillus megaterium* by ultraviolet irradiation and found that the resulting lysate was very active in promoting the lysis of all

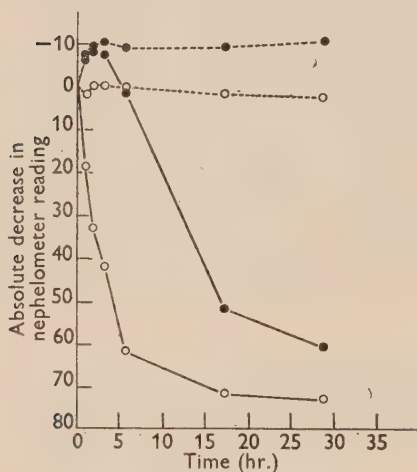


Fig. 5

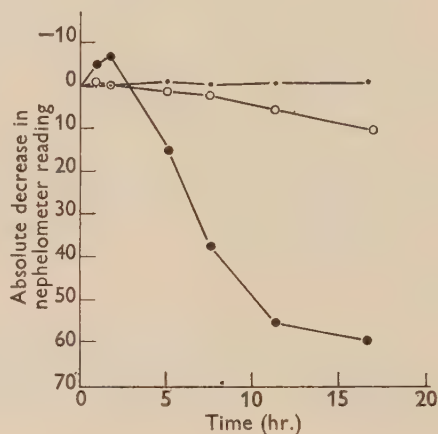


Fig. 6

Fig. 5. The action of the *Bacillus cereus* lytic principle on heated and unheated vegetative *B. sphaericus*. Unheated organisms suspended in lytic principle, ●—● and in heat-inactivated principle ●---●. Organisms heated at 80° for 20 min. and suspended in lytic principle ○—○, and in heat-inactivated principle ○---○.

Fig. 6. Vegetative *Bacillus sphaericus* suspended in lytic principle ●—● with 1/20,000 thiomersalate, and ○—○ without thiomersalate. ●---●. Organisms suspended in heat-inactivated lytic principle + thiomersalate.

strains of this organism tested. The agent was released during the lysis of the organism which produced it, was bactericidal and had properties indicative of a protein-like nature; it was largely species-specific. Greenberg & Halvorson (1955) demonstrated a lytic agent in old fluid cultures of *B. terminalis* (*B. cereus*). This agent was also largely species-specific, attacking only *B. cereus* strains, and had protein-like properties. Similar agents were demonstrated in two strains of *B. cereus* and *B. polymyxa*. Neither of these papers described the cytological changes which accompanied lysis. Boroff (1955) with the anaerobe *Clostridium botulinum* demonstrated in old cultures a lytic agent which was active against young actively growing cultures of the same organism. Lysis was followed microscopically with Gram-stained organisms; the series of changes seen closely resembled those described in the present paper. Cell-wall stains were not used, however, and the presence of cell-wall material at the end of lysis in these experiments must remain problematical. Boroff's agent was not characterized but was destroyed by boiling for 5 min.

The lytic principle described in the present paper resembles those investigated by the above workers in its main properties, which are consistent with its being protein in nature. The conditions under which it is formed and its marked non-specificity, however, serve to distinguish it from them. One cannot put forward more than tentative suggestions as to the mode of action of the agent. The nature of the lysis, the marked non-specificity and the ability to act on non-viable organisms speak against a phage mechanism, a conclusion supported by failure to demonstrate any increase in lytic activity during lysis of the substrate organism. Lysis might well be the result of the combined action of more than one constituent of the preparation, one possibly acting on the cell walls in such a way as to facilitate the entry of an actively lytic component into the cell. Concerning the role of thiomersalate, at the concentration used it causes little protein denaturation (McCulloch, 1945) and its activity in facilitating the action of the lytic principle may be attributable to interference with the normal metabolic processes of the organism, resulting in an enhanced susceptibility to the agent. Greenberg & Halvorson (1955) obtained a correlation between decrease in viable count and decrease in turbidity, attributing both effects to the action of their lytic agent. It should be borne in mind that preparations from organisms of this type may well possess additional antibiotic substances (Snell, Ijichi & Lewis, 1956) and a direct correlation between lysis and bactericidal activity need not necessarily exist. In the case of the agent here described the bactericidal action against *Staphylococcus aureus* was not associated with lysis; the two effects must be distinguished. In addition to their more general applications these observations serve to emphasize that decrease in optical density, which has often been used as an indicator of lysis, is a criterion which must be interpreted with caution since, although the contents of the cells are apparently going into solution, it does not necessarily follow that the cell walls are being disintegrated.

The author is indebted to Mr J. Wolf, Head of the Bacteriology Section for advice and criticism and to Mr G. W. Ripley of the Botany Department for the electron micrographs. The work was carried out during the tenure of an Agricultural Research Council Research Scholarship.

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EXPLANATION OF PLATES

PLATE 1

Changes in the appearance of vegetative *Bacillus sphaericus* 1652 suspended in solution containing the lytic principle and 1/20,000 thiomersalate; incubated at 37°. Light microscope; $\times 1250$ approximately.

Fig. 1. Organisms at the beginning of lysis. Gram stain.

Fig. 2. Organisms after 2 hr. of lysis. Gram stain.

Fig. 3. Debris left at the end of 16 hr. of lysis. Gram stain.

Fig. 4. Washed deposit from the preparation in fig. 3. Cell-wall stain.

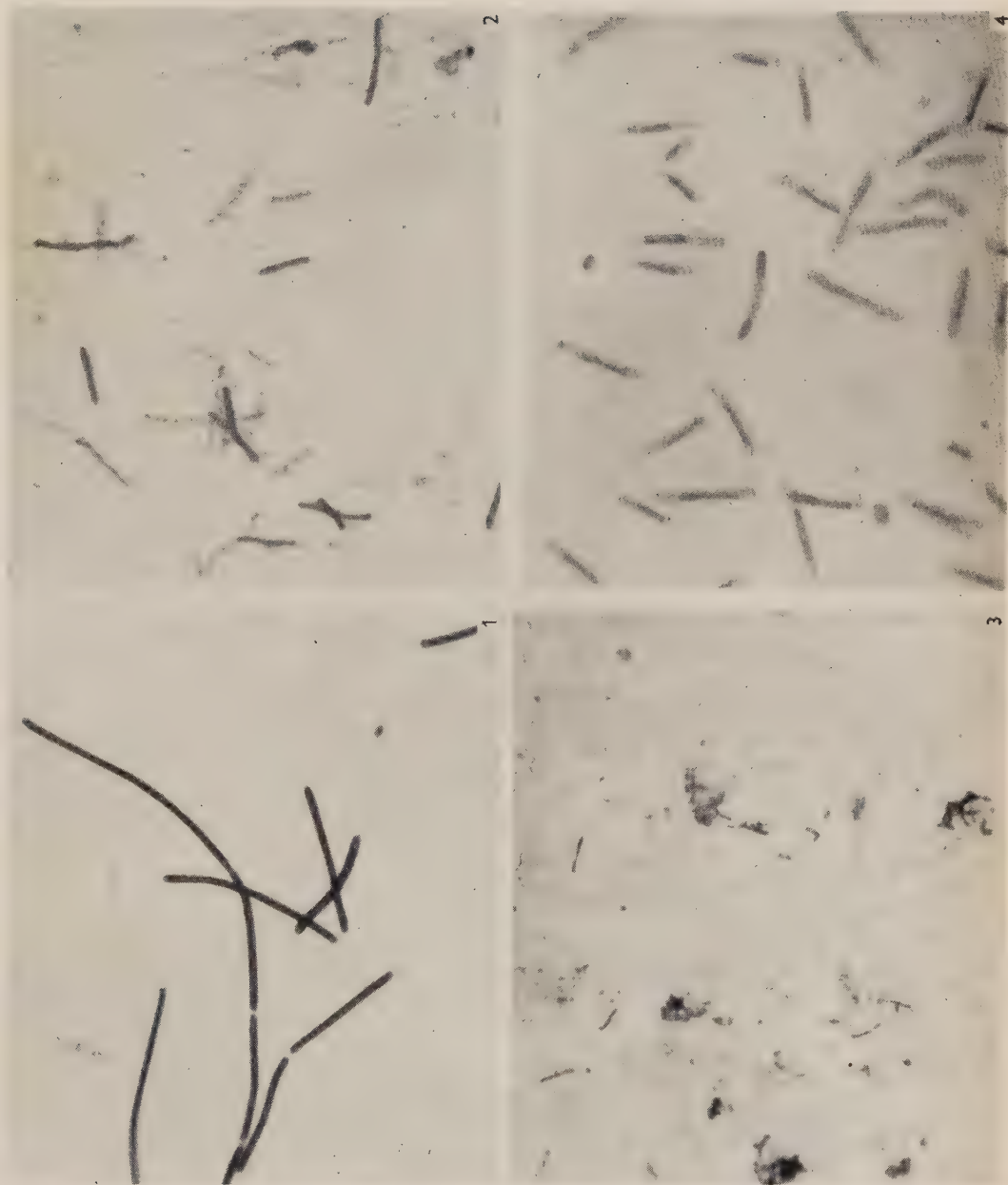
PLATE 2

Electron micrographs of vegetative *Bacillus sphaericus* 1652.

Fig. 5. Organisms suspended in heat-inactivated lytic principle + 1/20,000 thiomersalate and incubated for 16 hr. $\times 4300$.

Fig. 6. Organism suspended in active lytic principle + 1/20,000 thiomersalate and incubated for 16 hr. $\times 21,500$.

(Received 8 May 1956)



J. R. NORRIS—A LYTIC PRINCIPLE FROM *B. CEREUS*. PLATE 1

(Facing p. 8)



J. R. NORRIS A LYTIC PRINCIPLE FROM *B. CEREUS*. PLATE 2

HAYWARD, A. C. (1957). *J. gen. Microbiol.* **16**, 9-15

Detection of Gas Production from Glucose by Heterofermentative Lactic Acid Bacteria

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SUMMARY: Several modifications of the Durham tube method for detecting gas production have been tested with a variety of heterofermentative lactobacilli, some of which had been described in the literature as failing to produce gas from glucose by this method. Gas production in Durham tubes was detected consistently when the surface of a tomato juice broth medium containing 0.5 % or 2.5 % (w/v) glucose was sealed with liquid paraffin or with washed agar, but less consistently when the surface of the medium was unsealed. A minority of the strains examined grew weakly in a peptone + yeast extract medium containing glucose and produced very little titratable acidity or detectable gas. These produced large quantities of gas in the same medium when maltose replaced glucose.

Several methods may be used to differentiate between heterofermentative and homofermentative lactic acid bacteria. Analysis of the acid end products produced during the fermentation of hexoses shows that the former, including the genus *Leuconostoc* and some species of *Lactobacillus*, produce characteristic proportions of lactic and acetic acids, whereas the homofermentative species produce mainly lactic acid (Pederson, 1952). However, these two types may, for the most part, be differentiated by the production of gas from the fermentation of carbohydrates, which is confined to the heterofermentative organisms. This method, although simple in application, depends upon the reliability of the methods used for the detection of such gas (Davis, 1955*a*).

Many quantitative and qualitative methods of detecting gas production by lactic acid bacteria have been described (Gibson & Abd-el-Malek, 1945); qualitative tests have been carried out in liquid, solid or semi-solid media. The results of tests in liquid medium by the conventional Durham tube method, which is adequate for detecting gas production by Gram-negative bacteria, have led to erroneous conclusions. For example, Strålfors (1950) concluded that there were no gas-producing lactobacilli in the mouth, a conclusion at variance with the observations of Rogosa, Wiseman, Mitchell, Disraely & Beauman (1953) and Davis (1955*a*). The surface of the fermentation medium used by Strålfors was not sealed. The semi-solid medium of Gibson & Abd-el-Malek (1945) was sealed with a plug of nutrient agar, the displacement of which indicated gas production. Rogosa *et al.* (1953) detected gas production by the appearance of cracks in a 2 % (w/v) agar medium containing glucose + lactose; this solid medium was sealed with 2 % agar, the surface of which carried a thin layer of heavy mineral oil to prevent the escape of gas. Rosen, Ragheb, Hunt & Hoppert (1956) used a method similar to that described by Rogosa *et al.* (1953), in order to detect gas production by oral lactobacilli from rat. Although growth was observed in the medium, gas was produced in very small

quantities only in a few tubes. They thus concluded that most of the strains isolated were homofermentative, but their method clearly did not give unequivocal results.

Moore & Rainbow (1955), Camien, Dunn & Salle (1947) and Camien & Dunn (1955) described heterofermentative species of the genus *Lactobacillus* which fermented glucose very weakly, producing small quantities of titratable acidity, but which grew vigorously on a medium containing a pentose as energy source. Similarly, Cunningham & Smith (1940) isolated a group of lactobacilli from silage which gave low yields of fermentation products on glucose + yeast extract + tryptone broth. Although these organisms gave the low ratio of lactic to acetic acid characteristic of heterofermentative lactobacilli, the quantities of carbon dioxide produced were small, and in some cases comparable with the amounts yielded by homofermentative species. During the systematic study of numerous lactobacilli from diverse saprophytic and parasitic sources I have isolated several lactobacilli of this type which grew weakly or not at all upon a medium containing glucose as major source of energy. These observations led to the following re-investigation of the factors which affect the detection by the Durham tube method of gas production by lactic acid bacteria and to a search for a suitable fermentable substrate for gas production by those lactobacilli which ferment glucose weakly. Two experiments are described in the following paper: (i) gas production by known heterofermentative lactobacilli was compared in tomato juice (TJ) broth containing different concentrations of glucose with the surface of the medium sealed or unsealed, and also in TJ agar medium; (ii) gas production was compared in a medium containing various carbohydrates but no tomato juice.

METHODS

Strains L3, L4, L5 and L6, studied by Moore & Rainbow (1955), were supplied by courtesy of Dr C. F. Rainbow, Department of Applied Biochemistry, University of Birmingham. The remaining cultures were fresh isolates or were obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex; these are listed in Table 1.

Freshly isolated strains were identified according to a simplified form of the classification described by Davis (1955*b*). Those listed without specific names did not belong to any recognizable species.

Media. In the first experiment the tomato juice (TJ) medium of Davis, Bisset & Hale (1955) was used. Separate portions of this medium contained 0.5 % or 2.5 % (w/v) glucose; a third portion containing 2.5 % glucose and 0.5 ml./l. of a 2.0 % (w/v) solution of bromocresol green was solidified with 1.2 % (w/v) New Zealand agar. These media were dispensed in 3.0 ml. quantities in $\frac{1}{2} \times 5$ in. tubes. The tubes containing liquid media were supplied with Durham tubes, and were sterilized by autoclaving at 120°/15 min. The liquid media were inoculated with one drop of culture from a Pasteur pipette giving about 40 drops/ml. The molten and cooled agar medium was heavily inoculated with a Pasteur pipette and rotated to distribute the organisms evenly. The 2.5 %

glucose TJ liquid medium was inoculated with each strain in triplicate; one of the tubes was sealed with about 2.0 ml. of sterile liquid paraffin, one with a similar quantity of molten 10 % (w/v) washed Japanese agar, and the third tube was not sealed. The 0.5 % glucose TJ liquid medium was inoculated in

Table 1. *Classification and names of lactobacilli used*

Species and strain no.		Source
<i>Lactobacillus</i> sp.	L3, L4, L5, L6	Moore & Rainbow (1955)
<i>Lactobacillus</i> sp.	60C	Saliva
<i>Lactobacillus</i> sp.	68C4	Saliva
<i>Lactobacillus</i> sp.	S42/2	Wasp
<i>L. brevis</i>	C/2/1	New Zealand Cheddar
<i>L. brevis</i>	E2/2	Red Cheshire cheese
<i>L. brevis</i>	K/2/2	Tilsit cheese
<i>L. brevis</i>	I/4	Saint Paulin cheese
<i>L. brevis</i>	J/2	Canadian Cheddar cheese
<i>L. brevis</i>	23F/2	Saliva
<i>L. brevis</i>	S48/3	Dried yeast
<i>L. brevis</i> var. <i>rudensis</i>	4617	NCIB
<i>L. buchneri</i>	8037	NCIB
<i>L. fermenti</i>	V28	Human vagina
<i>L. fermenti</i>	74C4A	Saliva
<i>L. fermenti</i>	V8S	Human vagina
<i>L. parvus</i>	8516 (Russell & Walker, 1953 <i>b</i>)	NCIB
<i>L. malefermentans</i>	8517 (Russell & Walker, 1953 <i>a</i>)	NCIB
<i>L. frigidus</i>	8518 (Bhandari & Walker, 1953)	NCIB
<i>L. plantarum</i>	12C	Homofermentative species used as controls
<i>L. plantarum</i>	51C/1	
<i>L. casei</i>	15C/1A	
<i>L. casei</i>	76CF	
<i>Leuconostoc</i> sp.	N/2/1	Leicester cheese
<i>Leuconostoc</i> sp.	G/4	Dutch Edam cheese

duplicate; one tube was sealed with liquid paraffin, the other was not sealed. The following strains were incubated at 28° after inoculation with samples of 24 hr. TJ broth cultures: 8516, 8517, 8518, 8037, 4617, L3, L4, L5 and L6. All other strains were incubated at 37° after inoculation with 14 hr. cultures.

In the second experiment the following basal medium was used: Peptone (Oxoid) 0.5 % (w/v); yeast extract (Difco) 0.3 % (w/v); salts A 0.5 % (v/v); salts B 0.5 % (v/v); Tween 80 0.1 % (v/v); sodium acetate (hydrated) 0.5 % (w/v). The constituents were diluted in glass-distilled water and the pH value of the medium adjusted to 6.8–7.0. The salt solutions A and B were those used by Davis *et al.* (1955). Solution A contained 10 g. KH_2PO_4 and 10 g. K_2HPO_4 in 100 ml. of distilled water; solution B contained 11.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.4 g. $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ and 0.68 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml. of distilled water. (Note: These formulae are given in full because Davis *et al.*, in stating the composition of this medium, attributed both solutions to Rogosa *et al.* (1953), whereas the latter workers used only solution B).

Portions of the basal medium were supplemented with 2.5 % (w/v) of glucose, lactose, sucrose, fructose or maltose. About 3.0 ml. quantities were added to 0.5 in. \times 5 in. tubes containing Durham tubes (*c.* 1.2 in. \times 0.25 in.).

Sterilization was effected by autoclaving, momentarily, at 15 lb./sq.in. Tubes were inoculated with one drop of a 24 hr. TJ broth culture from a standard pipette. According to Moore & Rainbow (1955) their strains grew well at 32–35° in malt wort and so L3, L4, L5 and L6 were incubated at 34°, the remaining cultures at 36–37°. In sets of experiments tubes were incubated for 6 days without shaking, and examined daily for gas production.

RESULTS

The results of the two experiments are shown in Tables 2 and 3. In the unsealed tubes (Table 2), gas production was usually greatest after 2–3 days of incubation, the volume decreasing later presumably because of loss of gas to the

Table 2. *Expt. 1: gas production detected by the Durham tube method in tomato juice (TJ) media*

Gas production in liquid medium was recorded as the degree to which the Durham tube was filled with gas, as follows: 0 = Absence of gas in Durham tube; 1 = bubble of gas at apex of Durham tube; 2 = *c.* one-third of Durham tube filled with gas; 3 = *c.* two-thirds of Durham tube filled with gas; 4 = whole of Durham tube filled with gas. Gas production in agar was recorded as follows: ++ = transverse cracks in agar, agar split apart and forced up the tube; + = cracks in agar, but medium not forced apart; – = medium entire. N.I. = not inoculated. The numbers in brackets indicate the day of incubation at which maximum gas production was observed.

Strain no.	2.5 % glucose TJ broth			0.5 % glucose TJ broth		2.5 % glucose TJ agar
	Agar seal	Paraffin seal	Unsealed	Paraffin seal	Unsealed	
8516	4 (2)	4 (3)	4 (2)	> 2 (2)	> 2 (2)	++ (1)
8517	4 (3)	4 (3)	3 (3)	4 (6)	> 2 (3)	++ (2)
8518	4 (1)	4 (2)	4 (2)	> 3 (5)	3 (1)	++ (1)
4617	4 (1)	4 (1)	4 (1)	4 (3)	3 (1)	++ (1)
8037	4 (3)	4 (3)	> 3 (4)	4 (4)	0	++ (2)
L3	4 (2)	4 (2)	> 3 (3)	4 (3)	1 (2)	+(2)
L4	4 (2)	4 (3)	3 (2)	> 3 (5)	> 1 (3)	+(4)
L5	0	1 (3)	0	1 (3)	0	–
L6	1 (6)	1 (6)	0	1 (4)	0	–
V28	4 (2)	4 (3)	> 2 (3)	4 (4)	0	–
74C4A	4 (6)	> 2 (6)	0	1 (5)	0	–
68C4	4 (2)	4 (3)	3 (3)	4 (6)	0	+(5)
23F/2	4 (2)	4 (2)	4 (2)	4 (2)	> 2 (2)	+(4)
C/2/1	4 (2)	4 (2)	4 (2)	4 (3)	> 2 (2)	+(2)
E2/2	4 (1)	4 (1)	4 (1)	4 (2)	3 (2)	++ (1)
K/2/2	4 (2)	4 (1)	4 (2)	4 (3)	< 3 (2)	++ (1)
I/4	4 (1)	4 (2)	> 3 (2)	4 (3)	> 3 (2)	++ (1)
N/2/1	4 (3)	4 (3)	< 3 (2)	< 4 (3)	< 3 (2)	++ (1)
60C	4 (2)	4 (2)	4 (2)	> 3 (3)	> 2 (2)	++ (2)
G/4	4 (2)	4 (2)	> 3 (2)	4 (3)	> 1 (2)	++ (1)
J/2	4 (1)	4 (2)	4 (2)	4 (3)	> 2 (2)	++ (1)
15C/1A	0	0	1 (6)	0	1 (1)	–

atmosphere. Durham tubes filled with gas were forced into the liquid paraffin seal or agar plug, the latter being forced up the tube by the pressure of gas not collecting in the Durham tube.

In Expt. 1 (Table 2) homofermentative strains 12C, 51C/1 and 76CF, and in Expt. 2 strain 51C/1, did not produce detectable gas. As shown in Table 2, *Lactobacillus casei* (15C/1A) produced a very small quantity of gas in TJ broth, comparable with that produced by L5 and L6 in the same type of medium. In Expt. 2 (Table 3), however, L5 and L6 produced gas in large quantities in maltose medium. It has been observed in routine examinations that some strains of the homofermentative species *L. casei* and *L. plantarum* occasionally produce a bubble of gas at the apex of the Durham tube, but never in quantities comparable with those produced by heterofermentative species.

Table 3. *Expt. 2: gas production by lactic acid bacteria from various carbohydrates in a peptone + yeast extract medium*

The degree of gas production is recorded as in Table 2.

Strain no.	Glucose		Fructose		Maltose		Lactose		Sucrose	
	Agar seal	Paraffin seal	Agar seal	Paraffin seal	Agar seal	Paraffin seal	Agar seal	ffin seal	Agar seal	Paraffin seal
L3	4 (4)	4 (3)	3 (3)	3 (5)	4 (2)	4 (2)	0	0	0	0
L4	> 1 (5)	> 1 (6)	3 (3)	< 3 (3)	4 (2)	4 (2)	0	0	0	0
L5	0	0	3 (5)	> 2 (3)	4 (2)	4 (2)	0	0	0	0
L6	0	0	> 3 (4)	> 2 (3)	4 (2)	4 (2)	0	0	0	0
V28	4 (4)	4 (4)	4 (2)	4 (3)	4 (2)	4 (3)	0	0	4 (3)	4 (3)
V8S	4 (2)	4 (2)	< 3 (4)	> 2 (2)	4 (1)	4 (1)	4 (1)	4 (1)	4 (1)	4 (1)
S42/2	4 (2)	4 (2)	0	0	4 (2)	4 (2)	0	0	0	0
68C4	0	0	< 4 (4)	4 (5)	4 (2)	4 (3)	0	0	4 (2)	4 (3)
4617	4 (2)	4 (2)	3 (4)	3 (4)	4 (1)	4 (1)	0	0	4 (3)	4 (4)
S48/3	4 (3)	4 (3)	> 2 (2)	< 3 (3)	4 (2)	4 (2)	0	0	N.I.	N.I.
8518	4 (2)	4 (2)	4 (3)	4 (2)	4 (2)	4 (2)	0	0	4 (2)	4 (2)
8037	4 (5)	4 (4)	4 (2)	4 (2)	4 (3)	4 (4)	0	0	4 (3)	4 (3)

Strain 68C4, which in previous studies had failed to produce visible turbidity or titrable acidity in media containing glucose, also produced gas in a medium containing maltose, which according to Rogosa *et al.* (1953) is fermented by all heterofermentative lactobacilli. *Lactobacillus fermenti* (V8S) alone produced detectable gas in lactose medium. Gas production in fructose medium was generally less than in the other media.

Tables 2 and 3 show that very similar results were obtained in media sealed with agar or liquid paraffin; but in unsealed media gas accumulated in smaller quantities and was sometimes not detected. Results of tests in the agar medium used were frequently equivocal.

DISCUSSION

It is apparent that the Durham tube method may be used for the detection of gas production by lactobacilli provided that the surface of the medium be sealed, thus preventing the escape of gas, presumably mainly carbon dioxide. A minority of the tubes of unsealed media containing Durham tubes failed to accumulate gas. Evidence in the literature (for example, Strålfors, 1950)

suggests that in other hands all such tubes were free of gas; a possible explanation of this difference is that wider test tubes than those used in my experiments have been generally employed in gas production tests, with the consequent exposure of a larger surface area of medium to the atmosphere.

The use of tomato juice in gas-production media may give misleading results, bearing in mind the observations of Chatfield & Adams (1931), that tomatoes contain an average of 3.4 % total sugars: gas may be produced from carbohydrates present in the tomato juice, but probably not from glucose (compare the results for strain 68C4). It is noteworthy that the gas-production medium used by Gibson & Abd-el-Malek (1945) included skimmed milk, tomato juice and 5.0 % (w/v) glucose, while that of Rogosa *et al.* (1953) included tomato juice, 0.2 % (w/v) lactose and 0.3 % (w/v) glucose. Although such media are adequate for the detection of gas production by most heterofermentative lactobacilli, it is concluded from this study that some strains require differentiation from non-gas-producing lactobacilli upon a medium containing maltose, in which all the heterofermentative lactobacilli examined were more readily defined as such than in any other medium.

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Haemolytic Material from Aerobic Sporing Bacilli

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SUMMARY: Material from a strain of *Bacillus subtilis* which causes haemolysis was investigated. A fraction possessing the solubility properties of an organic acid appeared to be responsible for the haemolytic activity of the organism, but in the culture filtrate this material was associated with protein which modified its solubility properties considerably. Similar material was found in the culture filtrates of other haemolytic aerobic sporing bacilli.

Many members of the genus *Bacillus* are capable of causing haemolysis (Guillaumie, 1950). It is known that some species produce lecithinase (McGaughey & Chu, 1948) and the haemolytic activity of these species may be accounted for in part, or completely, by their production of this enzyme (Chu, 1949). Production of lecithinase in this genus is, however, apparently confined to *B. anthracis*, *B. mycoides* and *B. cereus* (McGaughey & Chu, 1948), and it seemed that an investigation of the haemolytic activities of those members of the group which do not elaborate lecithinase might be of interest. Büsing (1950) examined the haemolytic activity of a strain of *B. subtilis* and concluded that it was due to an esterase, but he did not in fact demonstrate such enzymic activity in his culture filtrates.

METHODS

Bacteria were grown in liquid media either in modified Monod T-tubes (van Heyningen & Gladstone, 1953) or in whirling flasks (Mitchell, 1949). Each litre of medium contained the following constituents: 2.0 g. NaNO_3 ; 0.5 g. KCl ; 1.0 g. K_2HPO_4 ; 0.5 g. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 0.02 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 40 ml. corn steep liquor diffusate; 12.5 g. glucose; 2.5 g. asparagine.

Production of haemolysin was tested for on plates of agar containing 0.5 % (v/v) of four-times-washed red blood cells of various species. The egg-yolk turbidity test for lecithinase activity was carried out according to McGaughey & Chu (1948). Haemolysis was measured either visually by observing the time necessary after addition of haemolytic material for a line on the far side of a 1 cm. test tube to become visible through the haemolysing suspensions of red cells, or by an electrochemical technique to be described elsewhere. This latter method could not be applied to the estimation of haemolytic material in the culture filtrates owing to interference by reducing substances in the media. Protein was determined by the biuret method according to Gornall, Bardawill & David (1949). Ultraviolet absorption spectra were determined in a Beckman model DU spectrophotometer.

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RESULTS

Forty-three strains of aerobic spore-forming bacilli were screened for haemolytic activity against red blood cells of rabbit, horse, sheep and man; the results are summarized in Table 1. The fourteen most active strains (as judged by the extent of the haemolysis zones) were selected, grown for 24 hr. in T-tubes, and their culture filtrates tested for activity against human red blood cells and

Table 1. *Distribution of haemolytic activity amongst the strains of Bacillus spp. tested*

<i>Bacillus</i> species	No. of strains tested	Red blood cells			
		Rabbit	Horse	Sheep	Man
		No. of strains giving positive haemolytic reactions			
<i>B. subtilis</i>	6	2	2	1	2
<i>B. pumilus</i>	6	6	5	3	4
<i>B. licheniformis</i>	6	6	1	0	1
<i>B. cereus</i>	7	7	7	7	7
<i>B. mycoides</i>	3	1	2	0	0
<i>B. megaterium</i>	4	0	0	0	0
<i>B. carotarum</i>	3	0	0	0	0
<i>B. brevis</i>	5	3	2	0	0
<i>B. circulans</i>	2	1	1	1	1
<i>B. alvei</i>	1	1	1	0	1
Total	43	27	21	12	16

for egg-yolk reaction. On the basis of these tests, ten strains were chosen as strongly haemolytic and unreactive in the lecithinase test and therefore useful for further investigation. One strain of *Bacillus subtilis* (strain 14) was particularly active and it was decided to attempt the isolation of the haemolytic material produced by this organism.

Partial purification of the haemolytic protein of Bacillus subtilis strain 14

The haemolytic material produced by *Bacillus subtilis* strain 14 was quantitatively precipitated by 4/5 saturation with ammonium sulphate. The precipitate from each litre of filtrate was dissolved in 200 ml. 0.05 M- Na_2HPO_4 , a slight insoluble residue being removed by centrifugation. The solution was adjusted to pH 4.5 and a precipitate appeared. This was centrifuged off and the clear supernatant fluid was found to be free from haemolytic activity. The precipitate was dissolved in the least possible volume of 0.15 M-trishydroxymethylaminomethane ('tris') at pH 8.0. The 'tris' solution was made 10 mM with respect to Zn^{++} by the addition of 0.12 M- ZnCl_2 and the resultant inactive precipitate centrifuged down. This Zn^{++} precipitation brought about a twofold increase in the haemolytic activity per mg. protein in the supernatant fluid. The supernatant fluid was then made 20 mM with respect to ethylenediamine tetra-acetate (EDTA) and 3 vol. of saturated ammonium sulphate were added. The resultant precipitate was dissolved in 0.15 M- Na_2HPO_4 . This preparation was only about 6-7 times more active per mg.

protein than the original culture filtrate and was heterogeneous by zone electrophoresis on paper. However, further purification of this material was not attempted after the demonstration that the haemolytic material produced by this strain could be obtained in another form possessing very different solubility properties (see below).

The alcohol-soluble haemolytic material

The name tyrothricin was given by Hotchkiss & Dubos (1940) to the crude mixture of polypeptides (gramicidin and tyrocidine) from *Bacillus brevis* (strain B.G.) because of the similarity of this material to the bacteriostatic agent liberated by certain cultures of '*Tyrothrix*' (Rosenthal, 1925) which were later identified as strains of *B. subtilis* (Smith, 1941). It appeared possible that the haemolytic activity of the *B. subtilis* 14 culture filtrates might be due to surface-active polypeptides similar to tyrothricin (Dubos & Hotchkiss, 1941). The culture filtrates were therefore processed according to Dubos & Cattaneo (1939). Early experiments showed that haemolytic material could be obtained in this manner, but the differences between the properties of the resultant product and those of tyrothricin suggested various modifications. The method finally used was the following: 1.5 l. of culture filtrate was 80 % saturated with ammonium sulphate and the precipitate suspended for 2-3 hr. at room temperature in 75 ml. acetone, acidified with 0.2 ml. of 5 N-hydrochloric acid. The inactive protein precipitate was filtered off and the acetone solution evaporated at 45° under reduced pressure to c. 10 ml. This solution was then added to 5 vol. of water, the pH value adjusted to 3.5 and the resulting precipitate dissolved in the minimum volume of 'tris' buffer (pH 8.0). The material so prepared was thus distinguished from tyrothricin by its solubility in alkaline aqueous solutions. A further distinction was provided by the fact that, when the pH value of the 'tris' solution was lowered to 6.0, the haemolytic material could be extracted by ether, from which it could be re-extracted by alkaline aqueous buffer. It could be precipitated from alkaline aqueous solution by acidification or by adding alkaline earth cations, a characteristic which distinguished this material from the bacilipins (Newton, 1949). The barium-precipitated material was soluble in EDTA solutions or in ethanol. The acid-precipitated material was soluble in methanol, ethanol, *n*-butanol, acetone, diethyl ether and benzene, but not in light petroleum. The material absorbed in the long wavelength ultraviolet with a maximum at 275 m μ . (Fig. 1) and from this characteristic and its solubility properties is referred to as '275-acid'. The nature and the degree of purity of this preparation of '275-acid' are unknown; it gives a slowly developing biuret reaction and a weak ninhydrin reaction (5 mg. '275-acid' = 5 μ g. leucine), and its nitrogen content is c. 1 % (Kjeldahl N/dry wt.).

Relationship of the two forms of the haemolytic material

When the haemolytic protein fraction, at any stage of purification, is subjected to the procedure outlined in the previous section '275-acid' is obtained. Table 2 shows that as the purification proceeded the ratio of

'275-acid' to total u.v.-absorbing material greatly increased and it therefore appears that the haemolytic activity of the protein fractions is attributable to their content of '275-acid', the solubility properties of which must be greatly modified by its association with protein.

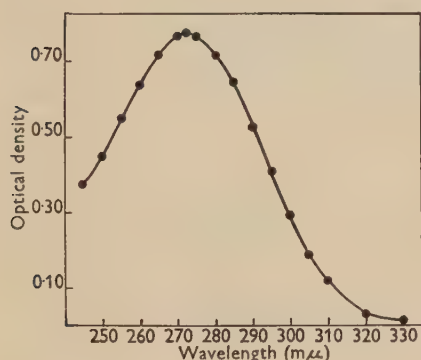


Fig. 1

Fig. 1. Absorption spectra of the alcohol-soluble haemolytic material. The $E_{1\text{ cm}}^{1\%}$ at 270 mμ. is 1.2.

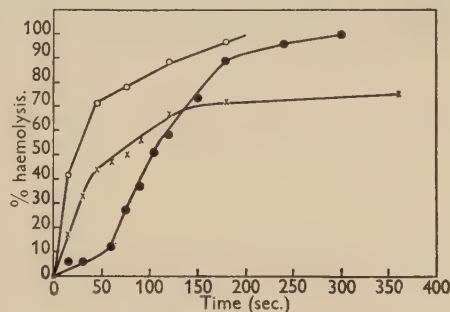


Fig. 2

Fig. 2. Kinetics of haemolysis by the '275-acid' preparation. ○—○, '275-acid'; 0.86 mg./ml. ×—×, '275-acid'; 0.35 mg./ml. ●—●, saponin; 0.07 mg./ml.

Table 2. The '275-acid' content of the protein fraction during purification

Fraction	Contribution of '275-acid' to total absorption at 275 mμ. (%)
Ammonium sulphate ppt.	13
pH 4.5 precipitate	32
Zinc-soluble	79

Biological distribution of '275-acid'

Culture filtrates from seven other haemolytic strains of aerobic spore-forming bacilli were examined for alcohol-soluble, acid-insoluble material absorbing at 270 mμ. The degree of haemolysis was measured by the visual method and as the relationship between dilution and rate of haemolysis appeared to be exponential under these conditions and the method itself is only semi-quantitative, it is not possible to give an accurate account of the haemolytic activities of these filtrates in terms of their content of '275-acid'. However, Table 3 clearly suggests that the haemolytic activity of the culture filtrates of *Bacillus licheniformis* 64 and *B. circulans* 82 cannot be attributed to the '275-acid' content of the filtrates, and that in other cases there may be other haemolytic material present. This question obviously requires much further examination.

Kinetics of haemolysis by '275-acid'

Fig. 2 shows the haemolysis progress curves given by the preparation of '275-acid' and washed human red blood cells. The S-shaped curve given by many haemolysins was not obtained and the shape of the curve resembles that obtained with tyrocidine (Bernheimer, 1947). However, the initial rate of haemolysis appears to be directly proportional to '275-acid' concentration, in which respect it differs from tyrocidine. It should, however, be observed that

Table 3. *Biological distribution of '275-acid' among Bacillus strains*

<i>Bacillus</i> species	Strain	Content of '275-acid' in culture filtrate as % of the content indi- cated by haemolytic activity
<i>B. subtilis</i>	14	100
<i>B. alvei</i>	G16	129
<i>B. subtilis</i>	119	120
<i>B. subtilis</i>	156	88
<i>B. subtilis</i>	12	60
<i>B. pumilus</i>	131	32
<i>B. circulans</i>	82	7
<i>B. licheniformis</i>	64	4

under the conditions of the visual assay the relationship between haemolysis rate and dilution appeared to be exponential. In this case the time observed is the time to reach an unknown but presumably high percentage of haemolysis and this method therefore tends to measure the slow phase of the haemolysis curves of Fig. 2, a distribution which may perhaps account for the different mathematical relationship. It will be observed that the preparation of '275-acid' in its present state of purity has a very low activity compared with, say, saponin.

DISCUSSION

The haemolytic activity of the protein fraction obtained from *Bacillus subtilis* strain 14 may be attributed to the acetone-soluble, water-insoluble '275-acid' that can be derived from this fraction. At first sight the association between the acid and the protein might appear to be of some biological importance by providing an example of a quasi-toxic protein the activity of which may be attributed to a 'prosthetic group'. van Heyningen (1950) commented on the possibility that the oxygen-labile haemolysins may contain a surface-active prosthetic group with haemolytic activity. However, the demonstration that the bulk of the ultraviolet absorption of the active protein fraction is accounted for by '275-acid', together with the fact that the $E_{1\text{cm.}}^{1\%}$ of '275-acid' is roughly one-tenth that of most proteins, suggests that, in fact, this fraction must be made up of c. 1 % of protein and 99 % of 'prosthetic group' on a weight for weight basis. This being so, it is doubtful whether this association is specific and

it may be that a non-specific 'solubilization' of '275-acid' should be invoked. Tyrothricin was originally obtained in a protein-bound form (Dubos, 1939) and in this case also the nature and specificity of the association is unknown (Hotchkiss, 1944).

This problem was suggested to me by Dr W. E. van Heyningen, and I am greatly indebted to him for many helpful discussions. The work was carried out under a Medical Research Council Grant for assistance to him. I gratefully acknowledge the competent technical assistance of Miss P. Rowlands.

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The Preferential Suppression of Hyaluronidase Formation in Cultures of *Staphylococcus aureus*

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SUMMARY: When *Staphylococcus aureus* strain 524/SC/55 is inoculated from an overnight culture into fresh broth only small amounts of hyaluronidase are formed at first, but while growth proceeds at a constant exponential rate an increasing proportion of the cell protein is secreted into the medium as hyaluronidase. This increase in proportion continues for eleven generations. Thereafter, it remains constant even when the organisms are transferred to fresh medium. The organisms of the inoculum taken from overnight cultures are deficient in thiamine and a partial deficiency of this and other essential growth factors suppresses the proportion of hyaluronidase formed. The accumulation of α -aminobutyric acid in organisms from overnight cultures was demonstrated; the addition of this substance to cultures decreases the proportion of cell protein which is turned into hyaluronidase.

It was shown (Rogers, 1954) that when staphylococci grew exponentially in broth or in casein hydrolysate media, the appearance of hyaluronidase activity in the cultures at first lagged and then accelerated throughout the period of rapid growth, leading to a continuously increasing ratio of enzymic activity to mass of organisms. Attempts were made to exclude activation of pro-enzyme, inactivation of enzyme formed early in the cultures or delayed secretion, as explanations of the phenomenon. If it be accepted that the measured hyaluronidase activities represent the amount of enzyme formed, then the increase in ratio of hyaluronidase activity to mass of bacteria during growth of a culture shows that an increasing proportion of the cell protein is secreted into the culture fluid as enzyme. If rapid growth could be extended for a longer period than normally occurs in a shaken flask culture, it seems that this increase must sooner or later cease and the ratio of hyaluronidase activity to total mass of bacterial protein become constant, i.e. the amount of enzyme formed must eventually be a constant proportion of the growing organism. In the present work, staphylococci were allowed to grow rapidly in broth for periods of up to 24 hr. in a continuous culture apparatus. During this period the ratio of hyaluronidase activity to mass of growth at first increased and then, as expected, became constant. When the cocci from such cultures were then transferred to fresh medium, hyaluronidase continued to form a constant proportion of the cell-mass during growth.

In all the work to be described the inoculum was taken from overnight cultures in which rapid growth had ceased for some time. It has now been found that cocci from such cultures are deficient in thiamine and that partial thiamine deficiency can decrease the proportion of hyaluronidase which is formed. It is suggested that this thiamine deficiency, together possibly with the accumulation of an inhibitor in the inoculum, might indirectly explain the slow acceleration to a steady rate of hyaluronidase formation.

METHODS

Organisms

The strain of *Staphylococcus aureus* originally isolated by selection of a single coccus from strain 524 (Rogers, 1953) and called 524/SC/55 was studied exclusively. At about 4-monthly intervals a broth culture of the organism was seeded on nutrient agar and after incubation the resulting growth was harvested and again dried in ampoules by the Stamp (1947) technique. The overnight cultures for inoculation of the experiments recorded in the present paper were prepared by suspending the contents of one such ampoule in the required volume of broth in a conical flask which was shaken at 35°.

Media. Wright broth (Wright, 1933) was preferred to casein hydrolysate or more chemically defined media since staphylococcal hyaluronidase was stable during incubation for periods of at least 6 hr. in this medium. Some destruction occurred when casein hydrolysate dilutions of hyaluronidase were similarly treated. The broth was sometimes buffered by the aseptic addition of sufficient sterile 50 % (w/v) solution of sodium β -glycerophosphate to give a final concentration of 2.5 %. The final glucose concentration of the media was increased to 0.25 % by the aseptic addition of 10 % glucose solution previously sterilized by autoclaving at 120° for 15 min.

Procedure for flask cultures

Sterile, conical flasks, of different sizes from 50 ml. to 1.0 l. and plugged with cotton wool, were one-fifth filled with medium. The medium was then inoculated with 0.15 ml./100 ml. of an overnight culture of organism; such an inoculum gave *c.* 10^6 cocci/ml. The inoculated flasks were shaken at the maximum throw of a shaker (Kantorowicz, 1951) at 35°. Subsequent work has shown that such a procedure did not result in equal availability of oxygen to solutions in the different sizes of flask, but that in some aeration was poorer

Table 1. *Oxygen availability determined by the rate of oxidation of 0.5 N-sodium sulphite containing 10^{-4} M-CuSO₄ at 35°*

The flasks were shaken at the maximum throw of the shaker. Samples (2 ml.) were taken into 2.0 ml. of 0.5 N-iodine. The residual iodine was determined by titration with 0.1 N-sodium thiosulphate.

Flask vol. (ml.)	Fluid vol. (ml.)	Oxygen solution (ml./hr./100 ml.)	K _L a*
50	10	53	140
100	20	53	140
250	50	30	81
1000	200	65	171

* K_La=absorption rate mmole/hr./l./unit concentration difference. See Finn (1954) for discussion of the advantages of this type of notation.

than in others. The availability of oxygen was measured by the rate of oxidation of 0.5 N-sulphite solution catalysed by 10^{-4} M-CuSO₄. Table 1 shows the results obtained by this method when applied to fluid of four flasks of

increasing size, each filled with one-fifth of its volume of 0.5 N- Na_2SO_3 and shaken under the same conditions as the cultures. Aeration was least good for 50 ml. in a 250 ml. flask and best for 200 ml. in a litre flask. It seems likely that the difference can be explained as a result of different ratios of surface to fluid volume in the flasks and to the degree of turbulence caused by shaking. The ratio was greatest for the smallest flask and the turbulence greatest for the largest. The differences in the degree of oxygen availability did not seem to affect the behaviour of the cocci in the experiments to be reported here, since the same results for growth and hyaluronidase formation were obtained in different-sized flasks. Calculations of the oxygen consumption by the organisms from the Q_{O_2} ($\mu\text{l. O}_2$ absorbed/hr./mg. dry wt.), determined whilst the organisms were growing in broth, showed that oxygen supply would not become limiting, even in the most unfavourable sizes of flask, until the region in which hyaluronidase formation ceased. The rates of hyaluronidase formation and growth were mostly measured at bacterial densities of about 20–50 % of that at which oxygen supply became limiting, and were thus not likely to be influenced by oxygen shortage.

Procedure for continuous culture

The continuous culture apparatus was similar to the 'bactogène' described by Monod (1950) and was totally enclosed in a warm room at $35^\circ \pm 0.2^\circ$. The supply of medium was fed by gravity into a constant head device (Marriott bottle) and from this again by gravity through 15 ft. of 1.5 mm. diameter capillary glass tubing into an enclosed dripper of wide (> 2 mm.) nozzle diameter. From the dripper the medium flowed into a revolving (200 r.p.m.) flask (5 in. diam.) containing 100 ml. medium. The volume of the culture was maintained constant by a suction tube bent to touch the surface of fluid within the flask. The rate of flow of the medium was varied by altering the vertical distance between the nozzle of the dripper and the level in the Marriott bottle, and was measured by counting the number of drops/min. Warm, wet, sterile air was pumped (3 l./min.) over the surface of the culture. The method of medium feed was selected for the rapid flow rates of the present work, from the many pumping and other possible methods, as the simplest, most controllable and least liable to variation. It is important that the capillary should be long and wide rather than short and narrow, in order to avoid blocking by fibres of cotton wool or other particulate material in the medium. Measurements of the oxygen solution rates by the sulphite oxidation method showed that a bacterial population equivalent to 1.5 mg. dry wt. bacteria/ml. grew without the oxygen demand exceeding the solution rate. The greatest densities of organisms used in the present work were equivalent to about 1.0 mg. dry wt./ml.; most of the experiments were at densities of about 0.3–0.5 mg. dry wt./ml. To study hyaluronidase formation during the rapid growth of staphylococci the apparatus was run so that the growth rate was 'internally controlled' (Novick, 1955). To do this 100 ml. medium in the flask of the apparatus were inoculated with 0.15 ml. of an overnight culture, the flask revolved and the air supply started. The medium flow was not turned

on until the culture had reached the required density as judged by optical opacity measurements. The flow of medium was then started and increased until the population density fell slightly; then by slightly decreasing the flow rate again a constant opacity could be maintained. Since any increase in the rate of flow of the medium tended to wash-out organisms the rate of division was maximal for the medium.

The operation of the bactogène as an internally compensated system without photoelectric devices to monitor the opacity of the culture obviously requires more care than for slow growth of organisms with a known limiting factor. Moreover, it is probably unsafe to regard the conditions in the continuous culture of staphylococci growing on complex media at minimum generation times as reaching a steady state during the experiments. Considerable fluctuation in the concentration of nutrients may be possible. If such fluctuations occurred, however, they did not affect the growth rate. Once the correct rate of flow of medium had been established, no further adjustments were necessary. In view of our inadequate knowledge of the biosynthetic processes necessary to form proteins and other complex molecules, it may be wiser to regard the steady state in cells as a condition reached for the concentration of all components of the cell in an infinite number of generations but not necessarily in existence after any finite number.

During continuous culture experiments, samples of 2–5 ml. were withdrawn aseptically at intervals through a sampling tube provided on the apparatus, cooled rapidly to 0–4°, measured for optical opacity and centrifuged in the cold. The supernatant fluids were then removed and stored at 0–4° under toluene for not more than 24 hr. before their hyaluronidase potency was assayed.

Analytical methods

Growth and hyaluronidase. These were estimated as previously described (Rogers, 1954). The results are expressed as mg. bacterial nitrogen/ml. and Turbidity Reducing Units (TRU)/ml. respectively.

Thiamine was assayed by the yeast fermentation method (Atkin, Schultz & Frey, 1939; modified by Kline & Friedman, 1947). The method was further modified for the conventional Warburg apparatus. Casein hydrolysate, pyridoxin, nicotinamide and salts were present as well as glucose in the suspension medium for the yeast. The manometers were gassed with argon for 1 hr. before starting the manometric readings. It was found to be important to dilute the standard thiamine solutions in 0.2 % gelatin (as suggested by Kline & Friedman, 1947) rather than in water, if consistent results were to be obtained. A straight line response was obtained for concentrations between 5 and 30×10^{-9} g. thiamine.

RESULTS

Fig. 1 shows the type of results obtained in the continuous culture experiments. In this particular experiment, the density of the culture was held constant at about 0.04 mg. bacterial-N/ml. and the mean generation time was 37 min. The hyaluronidase activity/ml. culture increased from about 2.0 TRU/ml. to

25 TRU/ml. during the first 2-3 hr. of the experiment. Since the population density of the culture was maintained approximately constant during this time, the increase in enzyme activity/ml. culture means that there was a corresponding increase in the amount of hyaluronidase formed/mg. new bacterial protein formed. Hence the proportion of the bacterial protein secreted into the medium as hyaluronidase, starting very low, increased as the run continued and finally became constant at a relatively high value, as indicated by the part of the enzyme curve in Fig. 1 which is parallel to the time

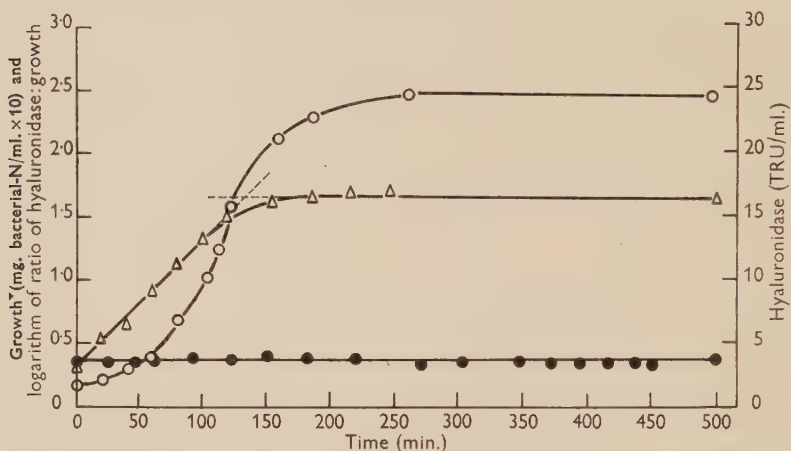


Fig. 1. The formation of hyaluronidase during a continuous culture experiment. ● = bacterial density (mg. bacterial-N/ml., $\times 10$). ○ = hyaluronidase activity (TRU/ml.). Δ = logarithm of the ratio hyaluronidase activity: mass of bacterial-N.

axis. Results similar to those shown in Fig. 1 were obtained in eighteen different continuous culture experiments. When the density of the culture was maintained at low values, the increase in proportion of hyaluronidase formed was usually greater, owing to the lower starting value, than at higher values (see Table 2). In three experiments, no increase in hyaluronidase occurred. These three experiments coincided with an attempt to replace the glass capillary tube on the medium feed of the apparatus by rubber tubing. It appeared that after two complete experiments during which the whole apparatus would be sterilized twice by autoclaving, the rubber tubing altered the medium in such a way that although the growth rate was as expected, no hyaluronidase was formed. The nature of the change brought about by rubber tubing has not been investigated.

Calculations were made of the total number of generations between inoculation and the point at which the organisms reached a steady rate of hyaluronidase formation (i.e. the point at which the enzyme formed a constant proportion of the bacterial protein of the growing cocci. This number of generations is made up of those which occur between inoculation and switching in the medium feed of the apparatus, and those which occur at constant bacterial density in the continuous culture. The curve for hyaluronidase potency does not flatten

abruptly, and some point had to be fixed arbitrarily in order to make the calculations. The time chosen was that of an intersection of the extrapolation of the logarithmic plot of the ratio of hyaluronidase activity: mass of growth during the first 2 hr. which was an approximately straight line, with the horizontal line for the steady value of the ratio finally achieved. This method of deriving the point is indicated by broken lines in Fig. 1. Table 2 shows the results of the calculations, obtained from six different experiments. Within

Table 2. *The number of generations required before hyaluronidase formation and growth proceed at the same exponential rate*

The organisms were grown at constant densities of organisms (M) until after the exponential rates of enzyme formation and growth became equal at time (T). The number of generations between the original inoculation of the culture with 7×10^{-4} mg. bacterial-N/ml. and time (T) was calculated.

Expt.	M (mg. bacterial- N/ml.)	T (min.)	m.g.t.* (min.)	Generations	Enzyme (E/G)†	
					Initial	Final
1	0.100	145	40	10.7	410	940
2	0.072	167	24	13.7	83	840
3	0.050	137	28	11.1	199	970
4	0.050	140	33	10.8	24	520
5	0.040	112	21	10.9	117	710
6	0.035	195	37	10.8	36	700

* Mean generation time.

† Ratio of hyaluronidase (in TRU/ml.) to mass of bacterial growth as mg. bacterial-N/m

very close limits five out of six of the experiments showed that a total of eleven generations had to elapse before the exponential rates of hyaluronidase formation and growth became equal (i.e. before hyaluronidase protein formed a constant proportion of the total). The considerable variation in the mean generation times of the organisms in the different experiments was caused by uncontrolled differences between different batches of broth. The one experiment in which a value different from eleven generations was obtained (Expt. 2) was unsatisfactory because an electrical failure stopped the apparatus for some time, and during further adjustments the bacterial density doubled. It seems likely that this may account for the difference between Expt. 2 and the others.

Re-growth of cocci from a continuous culture

The establishment of a steady rate of hyaluronidase formation which occurred eleven generations after inoculation of medium with cocci from an overnight culture might be due to a change in the fluid part of the culture or to a change in the organisms. If the latter had occurred, the organisms should continue to grow in a fresh medium with a constant ratio of hyaluronidase activity to mass of growth. To test this possibility, cocci introduced into fresh broth from a continuous culture which had been running for 6-7 hr. (equivalent to seventeen generations from inoculation) were compared with respect to growth and hyaluronidase formation with the behaviour of cocci taken from a shaken flask culture of the same opacity which had been inoculated in the

same way as the continuous culture but in which only four to five generations of growth had occurred. The details of the experiment were as follows. The continuous culture was maintained at an opacity equivalent to 0.036 mg. bacterial-N/ml. for 6 hr.; hyaluronidase determinations showed that the curve had flattened after the usual eleven generations (i.e. after 3 hr. 30 min. growth). The culture was cooled in ice water and 25 ml. of it centrifuged at 0–4°. At the same time as the medium flow of the continuous culture apparatus was started, a 500 ml. conical flask containing 100 ml. of warmed broth was inoculated with 0.15 ml. of the same overnight culture as used for the continuous culture. This flask was shaken until the opacity was equivalent to 0.036 mg. bacterial-N/ml. and then cooled. A sample (25 ml.) of this culture was also centrifuged in the cold. The two sets of cocci were then suspended, without washing, in 100 ml. of warmed broth in 500 ml. flasks, returned to the incubator and shaken. Samples (2.5 ml.) were removed initially and at 20 min. intervals during 3 hr.; the mass of growth and the hyaluronidase activities were estimated in the samples.

Difficulties were encountered in finding the most satisfactory way of analysing the results obtained. When the results for growth from a large inoculum and for the formation of any extracellular enzyme liberated into a fresh enzyme-free medium, are plotted logarithmically against time, they do not give parallel straight lines, even though a constant proportion of cell-material is liberated as enzyme. The device employed by Monod, Pappenheimer & Cohen-Bazire (1952), however, of plotting increase in enzyme activity/ml. culture [ΔE] against increase in mass of growth [ΔG] overcomes this difficulty and has proved satisfactory. When a constant proportion of the cell protein synthesized is turned into hyaluronidase, a straight line will result. When enzyme formation accelerates relative to growth, a curve convex to the increase in mass axis is obtained. Fig. 2 shows three comparisons of the behaviour of continuous culture organisms (Fig. 2*a*) with that of rapidly-growing shaken flask culture organisms (Fig. 2*b*). The continuous culture organisms formed a constant proportion of hyaluronidase (i.e. the ratio of hyaluronidase activity/mass of growth remained constant) and the plots of ΔG against ΔE are straight lines. The flask-grown organisms gave the expected convex curve, showing that the ratio hyaluronidase activity: mass of growth had increased. The cause of the considerable differences in the slopes of the straight lines obtained from the continuous culture organisms in different experiments is unknown. Similar results were obtained in two other experiments.

No sodium β -glycerophosphate was included in the media for the above experiments. Inclusion of this buffer in the continuous culture medium had no influence on the qualitative or quantitative aspects of hyaluronidase formation. When glycerophosphate was added to the media for re-growth of organisms in flask cultures, a slightly convex curve was obtained for the increasing growth: enzyme formation plot. The curvature was, however, very slight when compared with the results in Fig. 2*b*. It was now important to know how rapidly organisms which had divided eleven times reverted to organisms

which behaved like those from an ordinary overnight culture and which, when re-suspended in fresh medium, formed an increasing proportion of hyaluronidase during growth. A culture was grown for twelve generations in the continuous culture apparatus at a density of 0.05 mg. bacterial-N/ml. A volume (10 ml.) was then removed aseptically and placed in a 50 ml. conical flask and shaken at 35° for 14 hr. The density at the end of this

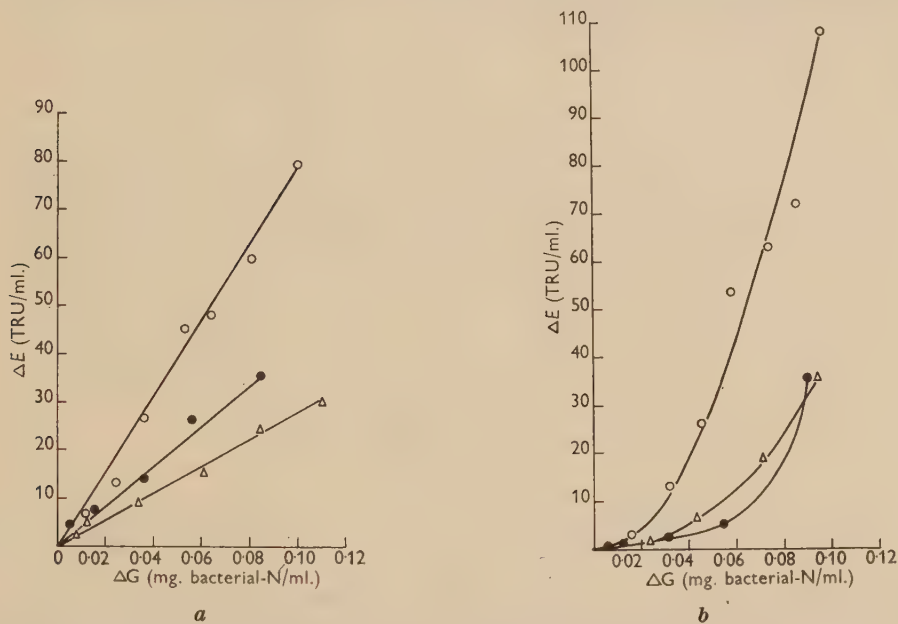


Fig. 2. Plot of the increase in growth (in mg. bacterial-N/ml., ΔG ; against increase in hyaluronidase (TRU/ml.), ΔE . Cultures grown in shaken flask cultures at 35°. ○, ● and Δ represent the results from three separate experiments. (a) Inoculum from a continuous culture. (b) Inoculum from a shaken flask culture.

incubation period was 0.6 mg. bacterial-N/ml. The cocci were separated by centrifugation and re-suspended in fresh medium containing no glycerophosphate to a density equivalent to 0.005 mg. bacterial-N/ml. Samples were taken at regular intervals and growth and hyaluronidase potency estimated as usual. Fig. 3 shows that the plot of the increase in mass of growth against hyaluronidase activity was again markedly convex, indicating that the cocci from the continuous culture had already reverted to the type of organisms present in an overnight culture during the four to five divisions during the incubation of the continuous culture cocci in a shaken flask. During these few generations, the growth rate gradually declined and almost ceased.

Selective suppression of hyaluronidase formation

The above findings suggested that the change during incubation of a culture overnight had affected the organisms in such a way that the general processes of protein formation necessary for growth and those for hyaluronidase

formation occurred at different rates when the cocci were inoculated into fresh medium. The rate of change of the organisms in both directions argues against a selection of mutants with differing ability to form hyaluronidase and in favour of some change in the majority of the population in the culture. Previously it was shown (Rogers, 1954) that during the late period of slow growth in an overnight culture no more hyaluronidase was formed. It now seems reasonable to argue that the cocci become progressively deficient in some substance during this period of growth.

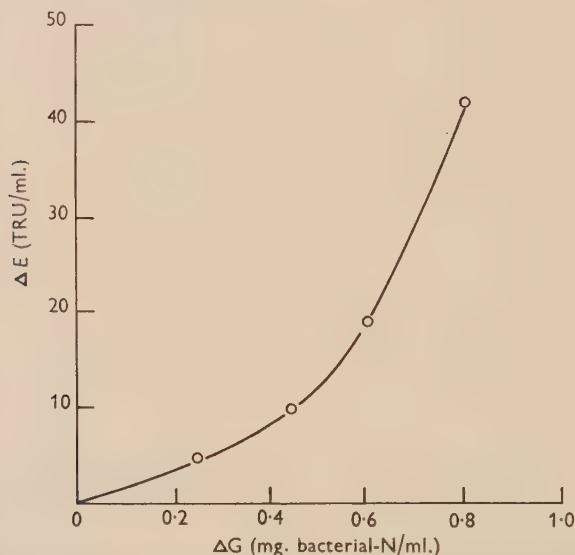


Fig. 3. Plot of the increase in growth (in mg. bacterial-N/ml.), ΔG against increase in hyaluronidase (in TRU/ml.), ΔE for a culture inoculated with cocci from a continuous culture which was removed from the apparatus and shaken overnight in a flask at 35°.

Thiamine deficiency

Early in the work it was found that the supernatant fluid obtained from a 'young shaken culture' in which the cocci were dividing rapidly would support growth of cocci from an overnight shaken culture, but that very little more hyaluronidase was usually formed. The details of an experiment demonstrating this are as follows. Two volumes (200 ml.) of broth supplemented with glycerophosphate, warmed to 35° and contained in 1 l. plugged conical flasks, were each inoculated with 0.3 ml. of an overnight culture. The flasks were incubated with shaking until the optical opacity of one of the cultures (A) was equivalent to 0.023 mg. bacterial-N/ml. This culture (A) was then cooled to 5° and centrifuged in the cold. The supernatant fluid was removed aseptically and stored at -2°. The second flask (culture B) was incubated overnight, then centrifuged and the organisms, drained of supernatant fluid, were re-suspended in 100 ml. fresh cold broth. Volumes (20 ml.) of the supernatant fluid from (A) were then pipetted into four 100 ml. plugged conical flasks and warmed to 35°. To two of these flasks 0.2 ml. of sterile glucose solution, 10 % w/v, was added.

A third pair of flasks contained fresh broth supplemented with glycerophosphate and warmed to 35°. All the flasks were inoculated with sufficient of the suspension of cocci from (B) to give a density equivalent to 0.015–0.02 mg. bacterial-N/ml. They were incubated with shaking for 5.5 hr., i.e. until hyaluronidase formation had ceased. The cultures were then cooled to 0–5°, their optical opacities measured, and then centrifuged. Hyaluronidase was measured in the supernatant fluids; Table 3 shows the results obtained. The increase in mass of organism in the supernatant fluid was about 40 % less than

Table 3. *Preferential suppression of hyaluronidase formation due to depletion of the medium*

The supernatant fluid (A) was obtained from a culture of *Staphylococcus aureus* growing logarithmically in broth medium and which had reached a density equivalent to 0.02 mg. bacterial-N/ml. The inoculum contained sufficient cocci from a culture incubated 18 hr. to give an initial density of 0.015–0.02 mg. bacterial-N/ml. The flasks (100 ml. conical flasks containing 20 ml. of fluid) were incubated with shaking for 5.5 hr. at 35°.

Medium	ΔG^*	ΔE^\dagger	$\Delta E/\Delta G$
Supernatant fluid A	0.133	6.3	47
Supernatant fluid A + glucose (0.1 %)	0.132	7.9	60
Fresh broth	0.213	171	810

* Increase in mass of growth in mg. bacterial-N/ml.

† Increase in hyaluronidase in TRU/ml.

that obtained in fresh broth, but hyaluronidase formation was decreased by 97 %. The addition of more glucose to the supernatant fluid had no significant effect. Nine different batches of broth were examined by the above technique; with one exception similar results were obtained. With five out of nine batches growth was 20–40 % less in the supernatant fluid than in fresh broth but hyaluronidase was more than 70 % less. In two batches growth was only 7–10 % less, whereas hyaluronidase formation was 80 and 90 % less. It seemed that the supernatant fluid from a 'young culture' was already partially deficient in some material necessary for growth and enzyme formation and that this deficiency selectively suppressed hyaluronidase formation.

A variety of known growth factors was then added to supernatant fluid obtained from young shaken cultures by the above technique. Organisms from an overnight culture were added and the cultures incubated until hyaluronidase formation had ceased. The results for growth and hyaluronidase estimations are shown in Table 4. It will be seen that thiamine was capable of restoring hyaluronidase-forming capacity to the supernatant fluid. Thus cocci from an overnight culture and supernatant fluid from a young culture together had insufficient thiamine, suggesting that thiamine was concentrated from the medium by the cocci from the overnight culture during the first few divisions. The cocci can, presumably, grow and form hyaluronidase at the expense of this store.

The amounts of thiamine in the cocci and supernatant fluid in a culture were measured at various stages of growth. Five amounts (200 ml.) of broth

Table 4. *Addition of substances to the depleted supernatant fluid*

Supernatant fluid from a logarithmically growing culture was prepared when the population density was equivalent to 0.02 mg. bacterial-N/ml. This supernatant fluid was dispensed in 10 ml. amounts in 50 ml. conical flasks and supplemented by the various factors. Volumes (0.1 ml.) of the solutions of supplements were added to each flask. The flasks were inoculated and incubated as in Table 3.

Supplement	ΔG		ΔE		$\Delta E/\Delta G$	
	I	II	I	II	I	II
Unsupplemented	0.234	0.217	32	30	137	139
Mixed amino acids*	—	0.171	—	20	—	117
Growth-factor mixture 1†	0.318	0.277	113	261	358	940
Growth-factor mixture 4‡	0.238	—	37	—	150	—
Growth-factor mixture 5§	0.226	—	30	—	130	—
Growth-factor mixture 6	0.246	0.271	113	182	460	670
1-Glutamine	—	0.165	—	15	—	91
Thiamine	—	0.271	—	206	—	760
Fresh broth	0.230	0.283	180	251	780	890

* A mixture of amino acids to imitate the composition of cow's milk casein.

† This mixture had the following composition: nicotinamide, 50 mg.; Ca pantothenate, 100 mg.; pyridoxin, 50 mg.; riboflavin, 5 mg.; thiamine, 50 mg.; glutamine, 100 mg.; *p*-aminobenzoic acid, 10 mg. Dissolved in 50 ml. water and sterilized by Seitz filtration.

‡ Contained nicotinamide, pyridoxin and *p*-aminobenzoic acid as in solution 1.

§ Contained Ca pantothenate and riboflavin as in solution 1.

|| Contained thiamine and glutamine as in solution 1.

containing 2.5 % sodium β -glycerophosphate and 0.25 % glucose, warmed at 35° in 1 l. flasks, were each inoculated with 0.3 ml. of an overnight culture grown in the same medium. When growth in the individual cultures reached the values indicated in Table 5, single flasks were removed from the incubator, cooled in ice water and centrifuged. A sample of the supernatant fluid was removed and the cocci washed once with 50 ml. distilled water. The washings were retained for thiamine assays in the first experiment but were subsequently rejected since none was detected in them. The cocci were then suspended in 10 ml. H₂O, the opacity of the suspensions measured and N-HCl added until they were acid to congo red. The suspensions and supernatant fluids (also acidified) were then heated for 30 min. at 100° to liberate thiamine (Kline & Friedman, 1947), neutralized to pH 6.0 with N-NaOH and adjusted to a volume of 25 ml. In some experiments the suspensions were subsequently treated with acidified sulphite solution (Schultz, Atkin & Frey, 1942) which alters the thiamine so that it will no longer assay by the yeast method but which leaves unaltered the free pyrimidine moiety which also, at higher concentrations, stimulates yeast and might confuse the estimations. Such experiments showed that about 75–95 % of the activity of the coccal suspensions in the yeast test was due to thiamine. In the supernatant fluids about 50 % of the activity remained after sulphite treatment. Hence these latter values are much less certain. As far as could be judged, however, no alteration took place in the ratio of sulphite-destructible to sulphite-stable activity during the growth of the organisms.

Table 5 shows that the amount of thiamine/mg. bacterial-N was highest in

the first sample in the recorded experiment. Thereafter the concentration of the growth factor in the cocci fell. A calculation of the expected concentrations of thiamine, made by assuming that all the growth which occurred after the first sample had been taken was at the expense of that stored in the cocci, shows that small amounts of thiamine continued to be removed from the fluid

Table 5. *The concentration of thiamine in growing cultures of Staphylococcus aureus*

Five conical flasks (1 l.), each containing 200 ml. medium, were incubated with shaking at 35°. One flask was removed, cooled and centrifuged at each of the times indicated in column 1. The cocci were washed and the thiamine in the cocci and in the supernatant fluid estimated as described in the text.

Time (hr.; min.)	Growth in culture (mg. bacterial-N/ml.)	Thiamine content in	
		Cocci (μ g./mg. bacterial-N)	Supernatant fluid (μ g./ml.)
3 05	0.0071	2.4	0.15
4 20	0.0320	1.2	0.15
5 10	0.065	0.80	0.14
6 55	0.151	0.41	0.12
18 0	0.310	0.28	0.12
	Uninoculated broth		0.23

by the organisms. After c. 7 hr. of growth, for example, the mass of bacteria in the culture had increased just over twenty times, whereas the internal thiamine concentration had decreased only six times. The probability of a continued uptake of thiamine by the cocci is supported by the decrease in the external thiamine concentration. Rather more disappeared from the fluid than could be accounted for by the difference between the amount calculated on the assumption of simple dilution during growth and observed internal concentrations in the cocci, but the amounts in the supernatant fluid were small and the difference was near the experimental error of the method.

Specificity of thiamine deficiency

The above observations showed that partial thiamine deficiency in a culture of staphylococci in broth can selectively depress hyaluronidase formation. It was of interest to discover whether this was specific for thiamine or whether cocci partially deficient in other essential growth factors behaved similarly. Nicotinic acid or nicotinamide is another essential growth factor for staphylococci and the influence of deficiency of this factor was examined. For this purpose the organisms were grown on a casein hydrolysate medium (medium B; Rogers, 1945) without added nicotinic acid, and different amounts of nicotinamide added. In another experiment, different amounts of thiamine were added to the medium containing nicotinamide. The media were dispensed in 10 ml. amounts in 50 ml. conical flasks, and each inoculated with 0.1 ml. of a 1/10 dilution of twice-washed cocci prepared from an overnight broth culture of the organisms (i.e. cocci from 5.0 ml. culture were suspended in 50 ml. water

and 0.1 ml. taken). The inoculated media were incubated at 35° with shaking until active hyaluronidase formation had ceased. It will be seen (Table 6) that nicotinic acid deficiency, like shortage of thiamine, selectively suppressed hyaluronidase formation. When sufficient of either factor was present to support half maximum growth hyaluronidase formation was suppressed 70–80 %.

Table 6. *The suppression of hyaluronidase formation by partial nutritional deficiency*

The basal medium was casein hydrolysate (B) of Rogers (1945) without nicotinic acid and thiamine. For the results under (a) all the flasks contained 1.0 µg. nicotinamide/ml.; under (b) all flasks contained 0.02 µg. thiamine/ml. All flasks were inoculated with twice-washed cocci from an 18 hr. culture to the equivalent of 7×10^{-4} mg. bacterial-N. Incubation was for 16 hr. at 35° with shaking.

(a) Variation in the concentration of thiamine

Thiamine (µg./ml.)	Mass of growth (mg. bacterial-N/ml.)	Hyaluronidase (TRU/ml.)
0.02	0.344	90
0.01	0.270	39
0.007	0.152	15
0.005	0.104	< 2
0.003	0.0835	< 2
0	0.0312	< 2

(b) Variation in the concentration of nicotinamide

Nicotinamide (ug./ml.)	Mass of growth (mg. bacterial-N/ml.)	Hyaluronidase (TRU/ml.)
1.0	0.244	56
0.1	0.182	30
0.05	0.105	12
0.02	0.080	2
0	0.040	< 1

Suppression of hyaluronidase formation by α-aminobutyric acid

The presence of α-aminobutyric acid in cultures of staphylococci was reported by Woiwood & Proom (1950). This substance has been shown to inhibit the growth of other micro-organisms (Gladstone, 1939; Rowley, 1953; Friedman, 1956). The amino acids liberated from staphylococci by heating suspensions at 100° for 10 min. (Gale, 1947) were examined by two-dimensional chromatography with phenol/NH₃ and butanol/acetic acid/water as the two solvent mixtures. Three different kinds of washed cocci were examined: (a) cocci, from shaken-flask cultures, which had been multiplying rapidly; (b) cocci from a continuous culture run for 6 hr.; (c) cocci from overnight shaken-flask cultures. Approximately the same total amounts of amino acids from each set were applied to the chromatogram. In all three fluids aspartic acid, glutamic acid and alanine predominated. In the extracts from the cocci grown overnight, however, a large spot giving a bright purple colour with ninhydrin appeared in the correct position for α-aminobutyric acid. No other

spots appeared when a mixture of the extract and α -aminobutyric acid were chromatographed. Examination of the extracts on copper-impregnated paper (Crumpler & Dent, 1949) showed that the additional spot given by the extracts from cocci from overnight cultures was not a γ amino acid. It was provisionally identified with α -aminobutyric acid.

Addition of α -aminobutyric acid, but not of γ -aminobutyric acid, to final concentrations of 0.01 M and 0.005 M to the broth medium gave very variable but significant suppression of the ratio hyaluronidase:mass of growth when cultures were grown from organisms from an overnight culture; the decrease in the ratio of hyaluronidase activity:mass of growth varied from 11 to 50 %. No significant inhibition of growth was shown with these concentrations of α -aminobutyric acid. The effect has so far proved too variable for detailed study. It seems probable that the selective inhibitory effect of the endogenous α -aminobutyric acid may be superimposed upon the selective suppression of enzyme formation by partial thiamine deficiency of the organisms from overnight cultures used to inoculate the cultures.

DISCUSSION

It has been shown that staphylococci from cultures which have ceased to grow rapidly, when inoculated into fresh medium, secrete an increasing proportion of their protein as hyaluronidase for about eleven generations. Thereafter hyaluronidase forms a constant proportion of the protein synthesized even when the organisms are removed from their growth medium into fresh broth. It is also now known that certain partial nutritional deficiencies can selectively suppress the formation of hyaluronidase and that the inoculum cocci from the overnight culture are deficient in thiamine. Since nicotinamide deficiency causes the same behaviour as thiamine deficiency, it seems unlikely that there is anything specific about the thiamine effect. It may be suggested that any factor which is present in suboptimal amounts will selectively suppress the formation of hyaluronidase. The slow increase in the proportion of hyaluronidase formed during the first eleven generations is not likely to be directly connected with the thiamine deficiency, because when the organisms are placed in fresh medium, they rapidly concentrate thiamine and grow at the expense of this store. If hyaluronidase formation were directly connected with the concentration of thiamine in the organism, the proportion of enzyme formed should rise very rapidly and then fall. If, however, as seems more probable, the amount of hyaluronidase formed is controlled by the concentration within the organism of some other material which depends for its formation on the intervention of diphosphothiamine, then it is easier to understand the very long time required before the maximum amount of enzyme can appear. The substances which control enzyme formation are likely to be required also for the protein formation necessary for growth, and it may require eleven generations before their concentration rises high enough to saturate the hyaluronidase-forming systems.

Superimposed upon the effects of thiamine deficiency is the selective inhibition

of hyaluronidase by α -aminobutyric acid which accumulates in cells from old cultures. Other work (Gladstone, 1939; Friedman, 1956) has shown that the addition to media of certain amino acids (e.g. valine with *Bacillus anthracis* and alanine+leucine with an *Escherichia coli* auxotroph which requires isoleucine+valine for optimum growth) can reverse the inhibitory action of α -aminobutyric acid. Friedman (1956) suggested that α -ketobutyrate derived from the α -aminobutyric acid interferes with the biosynthesis of α -ketoisovaleric acid, which is a valine and leucine precursor. Therefore, the suppression of hyaluronidase formation by α -aminobutyric acid may be another example of selective suppression by partial deficiency of valine or leucine.

A theory suggested by the present work seems to be that some mechanism exists in the cocci for sorting out priorities for constitutive enzyme formation when a deficiency of certain essential growth factors limits protein synthesis. Possibly the formation of some endogenous inducer of hyaluronidase is being altered by this deficiency, but the lack of specificity of the effect argues against such a hypothesis. It seems more probable that there are a series of enzyme-forming sites with different requirements for the concentration of intermediates of protein synthesis. The formation of some enzymes may proceed with relatively low available concentrations whereas others may require much higher concentrations.

In conclusion, it may be speculated as to whether a similar situation may not apply in systems where specific 'toxigenic' factors have been fruitlessly sought, such as in the formation of lethal toxin by *Clostridium tetani* or lecithinase by *C. welchii*.

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Some Hypotheses on the Aetiology of Fatal Infections in Partially Resistant Hosts and their Application to Mice Challenged with *Salmonella paratyphi-B* or *Salmonella typhimurium* by Intraperitoneal Injection

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SUMMARY: Some hypotheses are considered which describe the aetiology of a fatal infection in a partially resistant host; i.e. a host which does not invariably die after inoculation with one bacterium. The hypothesis of independent action postulates that the mean probability per inoculated bacterium of multiplying to cause (or help to cause) a fatal infection is independent of the number of bacteria inoculated and, for a partially resistant host, is less than unity ($1 > p > 0$). It predicts: (1) that the slope, b , of the probit-mortality/log-dose curve will be 2.0 or less at the LD 50 point; (2) that, while hosts dying after inoculation with many LD 50 die as a result of the multiplication of many of the inoculated bacteria, most of those dying from 1 LD 50 or less do so following the multiplication of only one of the inoculated bacteria, regardless of the total number of bacteria inoculated. When a mixture of several equally virulent, distinguishable variants of a given pathogen are inoculated, fatal infections caused by the growth of one bacterium should result in the predominance of only one variant at post mortem. The hypotheses of maximum and of partial synergism postulate that inoculated bacteria co-operate so that the value of p increases as the size of the dose increases. They predict: (1) that the slope of the dose-response curve may be more than 2.0 at the LD 50 point; (2) that all observed fatal infections will be initiated by more than one bacterium and that consequently the inoculation of a mixed inoculum will lead to the predominance of several variants at post mortem.

Variants of *Salmonella paratyphi-B* which carried one of the three flagellar antigens, b , i , or e,h , were prepared by transduction. Variants of *S. typhimurium* which either fermented or did not ferment xylose were also prepared. In each case, the variants did not differ detectably in growth rate *in vivo*, or in virulence. The value of b was 1.8 for mice inoculated with *S. paratyphi-B* by intraperitoneal injection (LD 50 = 7.7×10^6 organisms). As predicted by the hypothesis of independent action, the relative frequencies of the variants in samples of heart blood obtained post mortem varied greatly from mouse to mouse when the dose was 1 LD 50 or less, and became progressively more uniform (and similar to the inoculum) as the size of the dose increased. The value of b was 0.66 for mice challenged by *S. typhimurium* by subcutaneous injection (LD 50 = 2×10^3 organisms); and challenge with a mixed inoculum gave similar results.

Despite this general conformity with prediction, far fewer mice than expected yielded only one variant at post mortem. This discrepancy possibly resulted from

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a terminal breakdown in resistance, which was demonstrated by experiment. It is concluded that these results are most economically explained by the hypothesis of independent action and that this hypothesis is probably applicable to many other infective systems.

Many hosts only respond consistently to challenge by a pathogenic bacterium when the challenge dose contains many bacteria. The purpose of this paper is to discuss in general terms some possible reasons why, usually, many organisms will cause death when one organism will not, and to describe some relevant experiments. We shall consider only systems in which death is due to infection resulting from the multiplication of at least some of the inoculated organisms.

The relationship between inoculated bacteria might take one of two extreme forms. Either they could be assumed to be acting co-operatively, death being a consequence of their joint action, or they could be regarded as acting independently, more than one bacterium usually being needed because the probability of a given bacterium being lethal is less than unity (Halvorson, 1935; Druett, 1952). The situation when the LD₅₀ dose contains many organisms is analogous to that of a poor marksman firing at a bottle. Since his aim is poor, the bottle is unlikely to have been broken after a small number of shots has been fired but if he persists he will probably hit the bottle eventually. A local observer might be aware that the bottle was broken by the action of one bullet. On the other hand, a distant observer, informed only of the total number of shots fired before the bottle broke, would not be able to exclude the hypothesis that the breakage was due to the accumulated stresses produced by all the bullets fired.

We describe (1) the hypothesis of independent action, and (2) hypotheses of synergistic action, including the hypothesis of maximum synergism (hypothesis of the Minimal Lethal Dose) and hypotheses of partial synergism. Antagonism (interference) is not considered. The predictions of the hypotheses are first given for hosts which do not differ in resistance, not because it is thought that all hosts are the same, but because the way in which they differ is unknown. Consequently, it is impossible to make precise predictions when variation in host resistances is present. Two experiments are described which should show which hypothesis is applicable to a given system; the predicted results are given in the statement of each hypothesis. In the first experiment, the shape of the dose-response curve is determined. In the second experiment, hosts are inoculated with a mixture of recognizable variants of the same pathogen in order to estimate the number of inoculated organisms which initiate each fatal infection.

Many previous workers have pointed out that inoculated bacteria or viruses appear to act independently after inoculation. This may not be so in the last stages of a fatal infection when a pathogen may have so reduced host resistance that organisms which are normally saprophytic invade the blood stream. It is shown in the last section of the Results that a fatal infection can lead to a terminal bacteraemia by inoculated organisms which otherwise would not be present in the blood. Such a breakdown is considered to be responsible for discrepancies between prediction and observation in our experiments.

We define p as the mean probability per inoculated bacterium of multiplying to such an extent that it kills a given host (or would kill the host if it were not killed by other inoculated bacteria).

The hypothesis of independent action

This hypothesis states that p is independent of d , the number of bacteria inoculated; i.e. the inoculated bacteria act independently, the fate of one bacterium being unaffected by the other bacteria in the inoculum. The probability of a given host surviving a dose of one bacterium is then $1-p$, the probability of surviving a dose of two organisms is $(1-p)^2$, and the probability of surviving a dose of d organisms is $(1-p)^d$. We are only concerned with situations where the LD 50 is a large number, in which case d is large and p is small. The expression $(1-p)^d$ is then approximately equal to the first term of the Poisson series, so that we may write

$$S = e^{-pd}, \quad (1)$$

where S is the probability of a given host surviving a dose of d organisms and e is the base of natural logarithms (Lauffer & Price, 1945).

As d is large there will be only negligible differences between the numbers of bacteria in replicate doses taken from the same suspension.

(1) *The determination of the slope of the log-dose/probit-mortality curve.* The hypothesis of independent action predicts that the dose-mortality relation is given by the first term of the Poisson series if the hosts do not differ in resistance, that is, if p is the same for all hosts. Some curves derived in this way

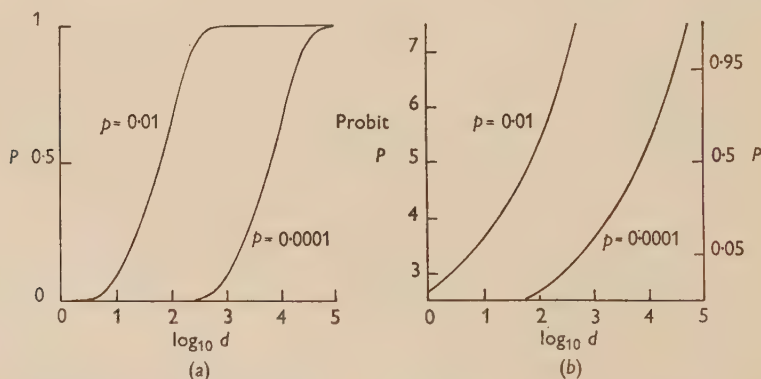


Fig. 1. Log₁₀-dose/mortality curves (a) and log₁₀-dose/probit-mortality curves (b) predicted by the hypothesis of independent action, when the hosts do not differ in resistance. P = proportion of hosts dying; d = mean number of viable bacteria per dose; p = mean probability per inoculated bacterium of multiplying to cause (or help to cause) a fatal infection.

are shown in Fig. 1 *a* and *b*, where P , the proportion of hosts killed ($= 1 - S$) and probit P , respectively, are plotted against the logarithm of the dose d . It can be seen that as p becomes smaller, that is, as the bacteria become less virulent, the curves shift to the right but their shapes remain the same.

It will be seen on the probit plot that, in the range of doses giving mortalities

between 5 and 95 %, the curves in Fig. 1*b* approximate to straight lines with the same slopes as those of the true curves at the LD 50 point. The term 'slope' will hereafter apply solely to the slope at the LD 50 point. Its value is 2.00 in Fig. 1*b* (Peto, 1953). When the hosts differ in resistance, the slope will be less than 2.00. It follows that where the hypothesis of independent action is applicable, the log-dose/probit-mortality curve will have a slope of either 2 or <2 . A slope of >2 would show that the theory of independent action was inapplicable.

(2) *The results of challenge by a mixture of recognizable variants of the same pathogen* (Kunkel, 1934; Lauffer & Price, 1945; Zelle, Lincoln & Young, 1946; Liu & Henle, 1953). This test is performed as follows. Two or more variants of the selected pathogen are isolated which differ only in some stable 'marker' character unrelated to virulence. For each variant the value of p and the rate of multiplication *in vivo* will be the same. The variants are mixed in equal proportions and hosts are challenged with graded doses of the mixture covering the range ≥ 1 LD 50 to <1 LD 50. All hosts that die are sampled to determine the composition of the terminal microbial population; in our experiments, heart blood was removed from each mouse. It is assumed that the bacteria present in the sample are the descendants of those inoculated bacteria which caused the fatal infection.

When, as has been assumed, $0 < p < 1$, only some of the bacteria in the challenge dose will multiply to contribute to the fatal infection. The probability that none will do so is given by the first term of the Poisson series when the hosts do not differ in resistance. Similarly, the probabilities that 1, 2, 3, ..., z of the administered bacteria will contribute to the fatal infection are given by the 2nd, 3rd, 4th, ..., $(z+1)$ th terms of this series. The probabilities that a fatal infection resulted from 1, 2 or 3 of the inoculated bacteria are plotted in Fig. 2 against dosage measured in multiples of 1 LD 50. It can be seen that at doses ≤ 1 LD 50, most of the fatally infected hosts die as a result of the multiplication of only one bacterium and each should yield a sample containing only bacteria with the same marker character. Hosts dying from the multiplication of more than one bacterium might also yield samples containing one variant, for two reasons. First, because only a small number of different variants can be used in practice so that, in a host dying as a result of the multiplication of, say, two bacteria, both bacteria might by chance carry the same marker character. Secondly, because it is unlikely that bacterial growth is synchronized *in vivo* and therefore one clone might produce a bacteraemia before another. It follows that the probability that a host dying from a small number of LD 50 will yield a sample containing only one variant is higher than the probability that such a host will die from the multiplication of one bacterium (the latter probabilities are those given in Fig. 2).

A host dying from a dose of many LD 50 will usually do so as a result of the multiplication of many bacteria and a sample from this host should contain the descendants of >1 bacterium. Since in practice only a few variants are used, the sample from such a host should contain all the variants in about the same proportions as in the challenge dose.

Since the variants are of equal virulence (i.e. have the same value of p), each has the same probability of being found in the sample from a given host dying from a small number of LD50 and each should appear with equal frequency when all the hosts are considered together.

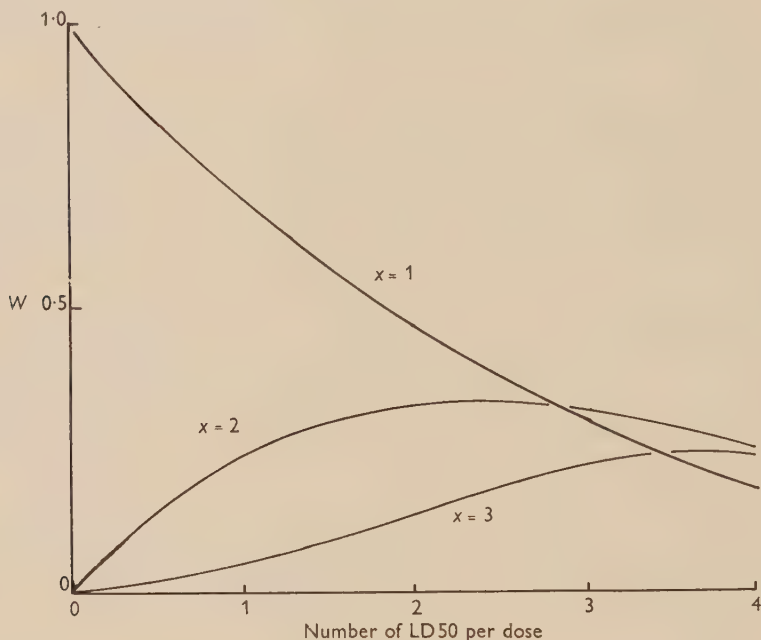


Fig. 2. Theoretical relationship between number of LD50's in dose and W , the probability that a given death resulted from the multiplication of x bacteria, for $x=1$, $x=2$ and $x=3$. Based on the hypothesis of independent action, when the hosts do not differ in resistance.

When the hosts differ from one another in resistance to a considerable extent, the predicted compositions of the samples will differ detectably from that shown in Fig. 2, according to the distribution of resistance amongst the hosts. The host population will consist of a mixture of unusually resistant and unusually susceptible individuals with a majority of average or near average resistance. When challenged by say, 1 LD50 dose, the more susceptible hosts will respond as if to a dose of more than 1 LD50, and will tend to yield samples containing all the variants. Conversely, when a dose of 10 LD50 is given, the more resistant hosts will respond as if to a dose of less than 10 LD50 and will tend to yield samples containing only one variant. The effect of variation in host resistance is therefore to increase the proportion of samples containing all the variants after challenge by small doses and to reduce the proportion after challenge by large doses.

The general prediction is, therefore, that so long as the hosts do not differ greatly in resistance, the compositions of samples from hosts challenged by large doses will be similar to that of the challenge dose and that they will differ

increasingly from the challenge dose, and from each other, as the size of the dose becomes smaller.

It has been assumed above that the appearance of one variant only in a sample is due to chance events following challenge and not to the selection of a clone of above-average virulence. It is possible, especially with an attenuated pathogen, that mutation to increased virulence might occur before or after challenge and that the bacterial population in a sample containing only one variant was descended from such a mutant. These possibilities can be distinguished by a control experiment. The strain appearing alone in a given sample is mixed with the strains of the other variants used in the original experiment and re-inoculated in graded doses into mice by the same route. If the strain appeared by chance as postulated, it should not predominate in the samples obtained in the control experiment; but if it was a mutant of increased virulence (or a mutant with a more rapid rate of growth *in vivo*), it should be consistently predominant.

Hypotheses of synergistic action

Having given the predictions of the hypothesis of independent action it is necessary to consider what results these experiments would give if the bacteria did not act independently, that is, if p varied with the size of the dose. We need only consider models in which synergism is assumed to occur so that p increases as d increases. The predictions of such models will vary according to the way in which p varies with d . An extreme case is the hypothesis of the minimal lethal dose (MLD; Finney, 1952), which in this context we may call the hypothesis of maximum synergism. This postulates that if the number of inoculated organisms is less (even by one organism) than the MLD for a particular host, none of the inoculated bacteria will multiply to cause a fatal infection ($p=0$) and the host will invariably survive; and that if the dose equals or exceeds the MLD all the inoculated organisms will multiply ($p=1$) so that the host will invariably die. In this case the slope of the log-dose/probit-mortality curve is a measure of the way in which the MLD varies in the host population; it will be infinite if the hosts do not differ in resistance, and may take any value between 0 and ∞ if they do vary. On this model all the inoculated bacteria multiply in hosts receiving at least 1 MLD and, if they multiply *in vivo* at the same average rate, the sample from each dead host will contain the same proportions of variants as did the inoculum.

Models involving partial synergism can be defined in which inoculated bacteria act independently at any given dose but where p increases as d increases. There will then always be a chance that death will be caused by only one of the many inoculated bacteria, and the likelihood that such infections will be detected experimentally is governed by the relationship between p and d and by the spacing of the doses. Partial synergism can only be distinguished from complete independence if the hosts do not differ in resistance.

It is obvious that the size of the LD 50 dose might be entirely determined by the failure of most of the challenge dose to penetrate the host, as during challenge by inhalation or application to the skin. This type of system seemed

to us less interesting than those in which the size of the LD₅₀ is entirely governed by processes occurring within the host tissues and for this reason the only method of challenge we have used is parenteral injection. In the first set of experiments, mice were challenged by intraperitoneal injection with *Salmonella paratyphi-B*, a system in which the LD₅₀ dose (without mucin) is about 10⁷ bacteria. It was thus possible to use a passaged strain of maximal virulence which possessed a large LD₅₀ value and which would be unlikely to mutate to increased virulence. The variants were prepared by transduction (Zinder & Lederberg, 1952) and differed in their flagellar antigens which, so far as is known, have no relationship to virulence. The experiments were repeated with two variants of an attenuated strain of *S. typhimurium* given by subcutaneous injection. Although mutants of increased virulence probably might have been isolated by passage, none was encountered during the experiments. The variants of *S. typhimurium* differed in their ability to ferment xylose; the composition of the sample populations was determined by inspection of the growth on plates of indicator medium.

METHODS

Preparation of marked strains and challenge doses. An O strain of *Salmonella paratyphi-B*, SW 666, was used; this strain is a galactose non-fermenting variant of strain SW 543 described by Stocker, Zinder & Lederberg (1953). It was first passaged three times in mice by intraperitoneal injection and from the passaged strain four H variants were prepared by transduction using the methods of Stocker *et al.* (1953). These variants were permanently fixed in phase 1 and carried the flagellar antigens e,h, r, i and b respectively. The first two variants were prepared using phage grown on *S. chester* (4, 5, 12: e,h; e,n,x) strain NCTC 5718, and an O strain of *S. heidelberg* (4, 5, 12: r; 1, 2. H antigens known to be potentially present in the O strain), SL 28, respectively. The variants carrying antigens b and i were both obtained by treatment with a lysate of *S. typhimurium* strain TM 2. The variant with antigen i resulted from transfer of an antigen-determining gene and a gene for flagellar production and that with antigen b (the latent antigen of strain SW 666) resulted from transfer of a gene for flagellar production only. None of these variants was lysogenic for phage PLT 22 used in their preparation. In a preliminary experiment the variant with antigen r formed an unexpectedly small proportion in the sample populations; it was therefore discarded. The other three variants (b, i and e,h) were passaged in mice a further three times, passed through tubes of semi-solid agar (Craigie, 1931) and finally freeze-dried. The challenge doses were prepared as follows:

The contents of a tube containing the variant in the dried state was suspended in broth and plated on three digest agar plates which were incubated overnight at 37°. Next day, the growth was suspended in 1/4 strength Ringer's solution and placed at 4° after a portion had been taken for the estimation of the viable count. The following day, when the count was known, appropriate volumes of the suspension of each variant were mixed to give a suspension of

known viable count containing equal proportions of the three variants. After further dilution in 1/4 strength Ringer's solution, a challenge dose of 0.5 ml. was given to each mouse by intraperitoneal injection.

In the second series of experiments the variants were prepared from *Salmonella typhimurium*, strain SW 351, a nutritionally exacting strain which fermented neither xylose nor galactose. After repeated treatments with phage lysates of a wild-type strain of *S. typhimurium*, TM2, a series of non-exacting variants differing in their power to ferment these sugars were obtained. The Xyl⁺ Gal⁻ variant had a shorter lag phase when grown in 1 % glucose peptone water than the other variants, although the exponential growth rates of the variants were the same. Therefore all challenge doses were prepared from suspensions kept in the exponential phase by the following method: Four blood agar plates, each bearing separate colonies of one variant, were stored in the refrigerator. On the first day of each experiment one colony of each variant was subcultured to one of four tubes containing 1 % glucose peptone water which was incubated at 37° overnight. On the second day, each culture was diluted 1/100 in fresh medium and incubated at 37° until it had been in the exponential phase for at least 1 hr., the rate of growth being estimated with a nephelometer. Each tube was then cooled to 10°–15° within 5 min. and then placed in the refrigerator. An exponential phase culture handled in this way is not killed and retains the ability to resume exponential growth immediately the temperature is raised to 37° (Sherman & Naylor, 1942). We have confirmed that this is true of our strain of *S. typhimurium*. When all the tubes had been at 4° for at least 30 min., the optical density (and inferred viable count) of each was measured. Appropriate volumes of the suspensions were then mixed to give a suspension containing equal proportions of the variants; challenge doses were prepared from this suspension by dilution in broth diluted 1/50 in chilled distilled water. Each mouse received 0.2 ml. chilled suspension by subcutaneous injection into the area overlying the left inguinal ligament.

In preliminary experiments the two Gal⁺ variants were found in a great preponderance in samples from mice which were dying after inoculation of mixtures of the four variants in equal parts; the Gal⁻ variants were therefore discarded.

The challenge doses in the third set of experiments, in which mice were challenged by a mixture of *Salmonella paratyphi-B* and *S. typhimurium*, were prepared by the method used with *S. paratyphi-B* alone. The strain of *S. typhimurium* (GLeB) was not related to the strains used in the second set of experiments.

In all experiments the viable count and composition of the challenge doses were checked by plating portions of the diluted mixed suspension to obtain separate colonies which were counted and scored.

Mice. The experiments with *Salmonella paratyphi-B* were performed on mice of the Webster BSVS line bred by brother-sister mating at the London School of Hygiene and Tropical Medicine. Those with *S. typhimurium* were performed on mice bred by random mating at the Postgraduate Medical School. The

weight of the former was 18–22 g. and that of the latter was 18–25 g. All mice were kept in large glass pots, not more than five mice being placed in each pot during the experiments with *S. typhimurium*; mice were allotted at random according to the tables of Fisher & Yates (1953).

Collection and scoring of samples. (a) Samples of heart blood. An effort was made to collect samples from mice when they were moribund rather than after death. This was difficult with mice infected by *Salmonella paratyphi-B* as death was not preceded by a period of visible ill health, whereas mice infected by *S. typhimurium* usually looked ill for at least 12 hr. before death.

Blood was withdrawn from the right ventricle and serially diluted in tenfold steps in either 1/4 strength Ringer's solution or 'Liquoid' broth (von Haebler & Miles, 1938), the dilutions thereafter being stored in the refrigerator. In the experiments with *Salmonella paratyphi-B*, a dilution of the samples was plated on nutrient agar plates to obtain discrete colonies. These were sub-cultured to thick plates of nutrient agar (0.9 % agar), incubated overnight at 22° and scored by slide-agglutination tests using specific phase 1 antisera.

Samples containing *Salmonella typhimurium* were inoculated by loop on to plates of indicator medium incubated overnight at 37° and the colonies scored as fermenters or non-fermenters by inspection. Control experiments with mixtures containing known proportions of the variants showed that this was an accurate method. The indicator medium had the following composition: 1/2 strength digest broth, 1000 ml.; xylose or galactose, 10 g.; sodium deoxycholate, 2.5 g.; neutral red, 0.025 g.

(b) Samples from abscesses. Mice surviving 3 weeks after challenge were killed by cervical dislocation. The skin overlying the abdomen was reflected and the abdominal wall seared. The abdominal cavity was opened with sterile precautions and the liver and spleen examined. Any abscesses found were removed, fresh instruments being used for each abscess, and after homogenization in a Griffith's grinder were examined in the same way as the samples of heart blood.

RESULTS

Experiments with Salmonella paratyphi-B

The LD 50 dose and the slope of the dose-mortality curve of each of the three variants (b, i and e,h) was estimated separately (Table 1); they did not differ significantly. The parameters of the dose-mortality curve (probit P plotted against $\log d$) of the summed results are $LD\ 50 = 7.7 \times 10^6$ (95 % confidence limits, $0.48-1.23 \times 10^7$) with slope, $b = 1.8$ (95 % confidence limits: 1.1–2.5). The observed value of b is therefore compatible with that derived from the hypothesis of independent action ($b \leq 2$). The value of p ($p = 0.67 \times 10^{-7}$, confidence limits, $0.48-1.0 \times 10^{-7}$) was estimated by the method given by Peto (1953).

The results of three experiments using mixed challenge doses are given in Table 2 which shows: the dose given to each group of mice; the number of mice dying; the proportion of variants present in the heart blood of each mouse; and two statistics, χ^2 and T , which indicate the extent to which the heart blood

Table 1. *Mortality in mice inoculated intraperitoneally with graded doses of three variants of Salmonella paratyphi-B; each variant tested separately*

Variant b					
<i>d</i>	1.62×10^8	5.12×10^7	1.62×10^7	5.12×10^6	1.62×10^6
<i>P</i>	5/5	4/5	4/5	0/5	1/5
LD 50 = 1.12×10^7 ; approximate confidence limits: $0.6-3.1 \times 10^7$					
Variant i					
<i>d</i>	1.16×10^8	3.67×10^7	1.16×10^7	3.67×10^6	1.16×10^6
<i>P</i>	5/5	5/5	4/5	2/5	0/5
LD 50 = 0.51×10^7 ; approximate confidence limits: $0.32-1.7 \times 10^7$					
Variant e,h					
<i>d</i>	1.42×10^8	4.49×10^7	1.42×10^7	4.49×10^6	1.42×10^6
<i>P</i>	5/5	4/5	5/5	0/5	1/6
LD 50 = 0.79×10^7 ; approximate confidence limits: $0.46-2.4 \times 10^7$					
Difference between slopes: $\chi^2 = 1.10$; 2 d.f.: $0.7 > P > 0.5$					

Parameters of the dose-response curve of the summed results:

LD 50 = 7.7×10^6 ; 95 % confidence limits $0.48-1.23 \times 10^7$.

$b = 1.81$; S.E. = 0.35.

$p = 0.67 \times 10^{-7}$; 95 % limits, $0.45-1.0 \times 10^{-7}$.

d: mean number of viable bacteria per challenge dose.

P: no. mice dying/no. mice inoculated (mice observed for 21 days).

samples have the same composition. Both χ^2 and T are derived from a comparison between the samples obtained from mice given the same dose. The value of χ^2 is shown with the number of degrees of freedom (d.f.) and the associated probability of obtaining such data by sampling from homogeneous populations. The second, $T = \sqrt{\{\chi^2/(N\sqrt{\text{d.f.}})\}}$, is Tschuprow's coefficient of association (Kendall, 1943) which enables one to compare the degree of homogeneity of different samples regardless of the numbers of observations and of degrees of freedom in each. This coefficient ranges between 0 and 1; the higher the value, the greater the degree of homogeneity.

The results of the three experiments, shown in Table 2, are broadly consistent with the predictions of the hypothesis of independent action; namely, that the compositions of the samples from all mice given many LD 50 doses should be similar to that of the challenge dose; that samples from mice given smaller doses should vary in composition from mouse to mouse; and that most samples from mice given 1 or < 1 LD 50 dose should each contain only a single variant.

It is clear from inspection in the first experiment that the samples from the 41.5 LD 50 group resemble each other more closely than do those from the mice given 4.15 LD 50 doses. None of the former have less than 16 % of any of the variants and there is a regular tendency for variant b to form a higher proportion in the samples than it did in the challenge dose. In the 4.15 LD 50 group, samples from mice 3 and 4 have only 9 % of variants i and b respectively; variant b does not regularly predominate and either e,h (nos. 1, 4 and 7) or i (no. 2) may predominate in some mice. Finally, the pair of samples from the

Table 2. *Mixed-inoculum experiments with Salmonella paratyphi-B: proportion of variants found in post-mortem blood of mice dying after intraperitoneal inoculation of a mixture of three variants*

Dose		Mortality (deaths/no. inoculated)	Mouse no.	Proportion of variant in post-mortem blood			No. colo- nies scored	χ^2	d.f.	<i>P</i>	<i>T</i>
No. viable bacteria	LD50			b	i	e, h					
Experiment 1											
3.2×10^8	41.5	5/5	(Inoculum	0.37	0.30	0.33	97)	11.5	8	> 0.1 < 0.2	0.120
			1	0.53	0.26	0.21	38				
			2	0.55	0.16	0.29	64				
			3	0.52	0.27	0.21	46				
			4	0.65	0.18	0.17	98				
3.2×10^7	4.15	8/8	5	0.40	0.30	0.30	37	68.3	12	< 0.001	0.236
			1	0.33	0.16	0.51	63				
			2	0.28	0.39	0.33	33				
			3	0.62	0.09	0.29	33				
			4	0.09	0.25	0.66	67				
			5	0.60	0.20	0.20	35				
			6	0.56	0.21	0.23	52				
			7	0.30	0.19	0.51	37				
1.6×10^7	2.07	3/8	8	Not scored			57.8	2	< 0.001	0.550	
			1*	0	0	1.0					101
			2	0.26	0.24	0.50					34
3	Not scored										
Experiment 2*											
1.54×10^9	200	5/5	(Inoculum	0.35	0.33	0.32	120)	4.6	8	< 0.8 > 0.7	0.105
			1	0.33	0.23	0.44	30				
			2	0.33	0.30	0.37	30				
			3	0.33	0.17	0.50	30				
			4	0.30	0.37	0.33	30				
7.7×10^8	100	5/5	5	0.23	0.30	0.47	30	13.3	8	0.1	0.177
			1	0.37	0.16	0.47	30				
			2	0.20	0.17	0.63	30				
			3	0.47	0.13	0.40	30				
			4	0.44	0.33	0.23	30				
3.85×10^8	50	5/5	5	0.30	0.20	0.50	30	6.3	8	< 0.7 > 0.5	0.122
			1	0.27	0.40	0.33	30				
			2	0.27	0.17	0.56	30				
			3	0.57	0.13	0.30	30				
			4	0.27	0.13	0.60	30				
1.92×10^8	25	5/5	5	0.30	0.13	0.57	30	19.1	8	< 0.02 > 0.01	0.212
			1	0.33	0.07	0.60	30				
			2	0.23	0.33	0.44	30				
			3	0.44	0.23	0.33	30				
			4	0.13	0.13	0.74	30				
5	0.17	0.20	0.63	30							

Table 2 (cont.)

Dose		Mortality (deaths/no. inoculated)	Mouse no.	Proportion of variant in post-mortem blood			No. colo- nies scored	χ^2	d.f.	P	T
No. viable bacteria	LD 50			b	i	e, h					
1.54×10^7	2	10/10	1	0.20	0.23	0.57	30	69.5	18	<0.001	0.234
			2	0.10	0.17	0.73	30				
			3	0.40	0.27	0.33	30				
			4	0.26	0.30	0.44	30				
			5	0.33	0.37	0.30	30				
			6	0.37	0.30	0.33	30				
			7	0.17	0.56	0.27	30				
			8	0.30	0.20	0.50	30				
			9	0.17	0.17	0.66	30				
			10	0	0.87	0.13	30				
7.7×10^6	1	6/10	1	0.20	0.23	0.57	30	23.5	8	<0.01 >0.001	0.194
			3	0.13	0.13	0.74	30				
			6	0.20	0.33	0.47	30				
			7	0.04	0.09	0.87	56				
			8	0.24	0.15	0.61	75				
			9	Not scored							
3.85×10^6	0.5	2/10	4	0.83	0	0.17	64	84.8	1	<0.001	0.82
			5	0.02	0	0.98	62				

Experiment 3

		(Inoculum		Not scored)			No. colo- nies scored	χ^2	d.f.	P	T
6.16×10^7	8	5/5	1	0.59	0.14	0.27	22	36.4	8	<0.001	0.258
			2	0.40	0.33	0.27	30				
			3	0.66	0.18	0.16	50				
			4	0.54	0.24	0.22	50				
			5	0.22	0.12	0.66	41				
7.7×10^6	1	6/10	1	0.22	0.50	0.28	36	116	8	<0.001	0.413
			2	0.53	0.22	0.25	36				
			3	0.29	0.39	0.32	31				
			4	0.50	0.16	0.34	36				
			5	0.02	0.96	0.02	99				
			6	Not scored							
3.85×10^6	0.5	3/10	1	0.36	0.36	0.28	34	134	4	<0.001	0.584
			2	0.04	0.81	0.15	123				
			3	0	0	1.0	40				
1.92×10^6	0.25	1/10	1	0.05	0.01	0.94	131

T is Tschuprow's coefficient of variation; $T = \sqrt{[(\chi^2/N\sqrt{d.f.})]}$. The values of χ^2 and of T are derived from comparisons between mice in each dose group.

* The inoculum mixture for Expt. 2 comprised the stock strains of variants b and i, combined with the e, h variant recovered in pure culture from mouse no. 1 of the group given 2.07 LD 50 in Expt. 1.

2.07 LD 50 group are even more heterogeneous owing to sample 1 containing only variant i in 101 colonies tested.

The strain of variant e, h isolated in pure culture from the first mouse of the 2.07 LD 50 group was then combined with original strains of variants b and i to prepare the challenge dose for Expt. 2, to test if it was a mutant of increased virulence or faster growth rate.

The results of Expt. 2 are similar to those of Expt. 1. Since variant e, h did

not regularly predominate, it was concluded that the strain isolated in Expt. 1 was not a mutant. For reasons given in the Discussion, we consider that the predictions of the theory of independent action are only tested by doses of *Salmonella paratyphi-B* ≤ 2 LD 50; nevertheless, the results with the higher doses used in this experiment show that the variants grew at the same rate in hosts whose resistance may, however, have been reduced by the large number of bacteria administered. Four samples from mice given lower doses contained over 80 % of one variant (2 LD 50, no. 10; 1 LD 50, no. 7; 0.5 LD 50, nos. 4 and 5). No samples containing only one variant were encountered.

The highest dose given in the third experiment was 8 LD 50. One sample contained only one variant (0.5 LD 50, no. 3, 40 colonies scored) and three contained a preponderance of one variant (1 LD 50, no. 5; 0.5 LD 50, no. 2 and 0.25 LD 50, no. 1).

The survivors in all experiments were killed 3 weeks after challenge and any isolated abscesses in the liver and spleen were removed and examined with the results shown in Table 3. Abscesses were rarely found in the spleen. Macroscopically normal liver was usually almost sterile; it is suspected that the exceptional specimen (Expt. 3, mouse *b*) contained a hidden abscess. Most abscesses contained about 10^6 viable bacteria composed in most cases of either a majority of or only one variant. One mouse (Expt. 1, no. 5) yielded ten abscesses of which eight contained a majority of variant *i* and one a majority of variant *b*, the observations on the remaining abscess (no. 9) being of little value because it was almost sterile and only three colonies were obtained.

Experiments with Salmonella typhimurium

As has been seen, the experiments with *Salmonella paratyphi-B* yielded fewer samples containing one variant than had been predicted from the hypothesis of independent action. We suspected that this was due to the effect of a terminal breakdown in resistance, caused by the bacteria initiating the fatal infection, which encouraged the multiplication of other bacteria to such an extent that they appeared in the sample. We supposed that this would be less likely to occur with a pathogen with a smaller LD 50 dose than that of *S. paratyphi-B*. The experiment was therefore repeated using *S. typhimurium* administered to mice by subcutaneous injection.

Table 4 gives the observations from which were computed the parameters of the dose-mortality curves of the Xyl⁺ Gal⁺ and Xyl⁻ Gal⁺ variants. These curves did not differ significantly when analysed by probit methods. The LD 50 doses were $10^{3.13}$ and $10^{3.42}$, the difference in log LD 50 being 0.29 (95 % confidence limits of -0.66 to +1.38) which was not significant, nor was there a significant difference in their slopes ($\chi^2=2.9$ on 1 d.f.; $P=0.09$). The mice clearly differed in resistance to a considerable extent for the common slope was 0.66 (95 % confidence limits: 0.34-0.98) which is significantly less than 2. For this reason it was not expected that samples containing one variant only would be encountered as predicted from the Poisson series in the case when the hosts did not differ in resistance. Nevertheless, the compositions of the

Table 3. *Mixed-inoculum experiments with Salmonella paratyphi-B: proportion of variants found in liver-abscesses, and in macroscopically normal liver, of mice surviving inoculation of a mixture of three variants*

In Expts. 1-3 (Table 2) mice surviving 21 days after inoculation were killed. Any liver abscesses seen were dissected out and cultured; portions of apparently normal liver were also cultured from some mice with abscesses.

Source of mouse (Table 2)			Specimen	Viable bacteria/ specimen	Proportion of variant in specimen			No. colonies tested
Expt. no.	Dose group	Mouse no.			b	i	e, h	
1	2.1 LD 50	5	Abscess 1	ND	0.17	0.63	0.20	35
			Abscess 2	ND	0.04	0.67	0.29	27
			Abscess 3	ND	0.05	0.95	0	61
			Abscess 4	ND	0	1.0	0	40
			Abscess 5	ND	0.13	0.84	0.03	32
			Abscess 6	ND	0	1.0	0	20
			Abscess 7	ND	0.10	0.90	0	20
			Abscess 8	ND	0.20	0.80	0	20
			Abscess 9	ND	1.0	0	0	3
			Abscess 10	ND	0.90	0	0.10	20
1	2.1 LD 50	6	Abscess	ND	0.84	0.16	0	38
1	1 LD 50	1	Abscess	ND	0	1.0	0	50
3	2 LD 50	a*	{ Abscess	1.6×10^6	0.54	0.46	0	39
			{ Liver	0	—	—	—	—
3	2 LD 50	b*	{ Abscess	1.65×10^6	0.97	0.03	0	36
			{ Liver	3.9×10^5	0	0.14	0.86	36
3	1 LD 50	c*	{ Abscess	3.6×10^5	1.0	0	0	39
			{ Liver	1.5×10^2	0.20	0.33	0.47	15
3	1 LD 50	d*	{ Abscess 1	1.75×10^6	0.20	0.77	0.03	39
			{ Abscess 2	9.4×10^5	0.62	0.16	0.24	34
			{ Abscess 3	9.5×10^5	0	1.0	0	36
			{ Liver	10^2	0	0	1.0	4

* Mice a, b, c and d were survivors from a subsidiary part of Expt. 3, not recorded in Table 2; the groups of mice concerned received the same inocula as the groups shown in Table 2, but in mice which died post-mortem blood was not cultured to determine the proportion of variants.

ND: viable count not done.

Table 4. *Mortality in mice inoculated intraperitoneally with graded doses of two variants of Salmonella typhimurium; each variant tested separately*

Xyl ⁺ Gal ⁺ variant							
d	6×10^4	2×10^4	6.6×10^3	2.2×10^3	7.3×10^2	2.4×10^2	8×10^1
P	6/6	6/6	3/6	4/6	3/6	3/6	1/6
LD 50 = 2.6×10^3							
Xyl ⁻ Gal ⁺ variant							
d	5.6×10^4	1.9×10^4	6.3×10^3	2.1×10^3	7×10^2	2.3×10^2	7.6×10^1
P	6/6	3/6	5/6	2/6	1/6	2/6	1/6
LD 50 = 1.3×10^3							

Difference in \log_{10} LD 50 = 0.29 (95 % confidence limits: -0.66 to +1.38).

Slope of the summed dose-mortality curves = 0.66 (95 % confidence limits: 0.34 to 0.98).

d: mean number of viable bacteria per challenge dose.

P: no. mice dying/no. mice challenged (mice observed for 21 days).

samples should have become increasingly heterogeneous as the size of the dose decreased if the hypothesis of independent action was applicable.

Fig. 3 shows the result of one experiment in which mice were challenged with a mixture of the Xyl⁺ Gal⁺ and Xyl⁻ Gal⁺ variants. The proportion of Xyl⁺ colonies in each sample is shown on the ordinate and the dose is given on the abscissa. Between 90 and 400 colonies were scored from each sample

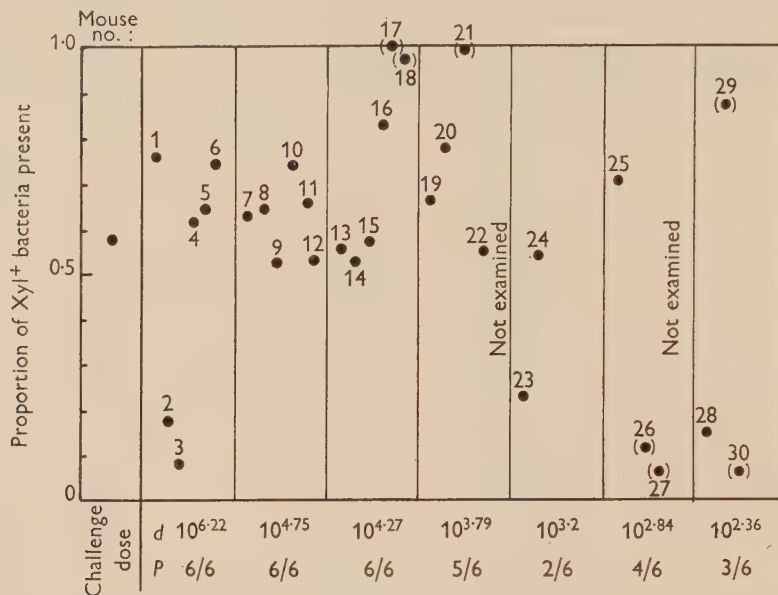


Fig. 3. Proportion of Xyl⁺ bacteria in samples of post-mortem heart blood of mice dying after inoculation of graded doses of a mixture of a Xyl⁺ and a Xyl⁻ variant of *Salmonella typhimurium*. Each dot represents the proportion of the Xyl⁺ variant found in blood from a given mouse (the composition of the inoculum is shown on the left). The recovered strains marked by brackets were tested in the control experiment recorded in Table 5. d: mean number of viable organisms per dose; P: number of mice dying/number of mice challenged.

except in five samples where the numbers were 71, 65, 34, 84 and 66. The results resemble those obtained with *Salmonella paratyphi-B*; the samples differ increasingly as the size of the dose becomes smaller. Also, samples were again encountered which had a great preponderance of one variant but fewer than expected contained only one variant. Each preponderant strain, from mice marked by brackets in Fig. 3, was recombined with the original strain of the alternative type and injected into four mice subcutaneously in a dose of 3.2×10^5 bacteria (about 100 LD₅₀ doses) to detect mutants. The results are given in Table 5 and show that a strain which was predominant in the original experiment never consistently predominated in the control experiment. Therefore these strains were not mutants. It is unfortunate that the variants from samples nos. 2 and 3 in the group given 10^{6.22} bacteria were not tested in this way, for these might well have been mutants. It can be seen that the samples in each group in the control experiment were heterogeneous. (The values of

Table 5. *Mixed-inoculum experiments with Salmonella typhimurium: control experiments on strains recovered, as predominant strains, from mice dying after inoculation of a mixture of two variants (Fig. 3)*

The predominant strain (Xyl⁺ or Xyl⁻) was recovered from each mouse marked with brackets in Fig. 3 and was mixed in equal proportions with the stock strain of the opposite Xyl character; each mixture was inoculated subcutaneously into four mice (dose *c.* 3×10^5 bacteria = *c.* 100 LD 50). Post-mortem heart blood from these mice was plated to determine the proportion of the variants; each inoculum mixture was similarly plated.

Recovered strain			Proportion of		No. colonies scored	χ^2	d.f.	P	T
Source: Fig. 3, mouse no.	Xyl character	Mouse no.	Xyl ⁺	Xyl ⁻					
17	Xyl ⁺	(Inoculum	0.58	0.42	136)	210.5	3	< 0.001	0.515
		1	0.57	0.43	89				
		2	0.93	0.07	111				
		3	1.0	0	150				
		4	0.79	0.21	109				
18	Xyl ⁺	(Inoculum	0.62	0.38	103)	171.5	3	< 0.001	0.455
		1	0.80	0.20	189				
		2	0.11	0.89	126				
		3	0.49	0.51	100				
		4	0.90	0.10	63				
21	Xyl ⁺	(Inoculum	0.67	0.33	85)	110.3	3	< 0.001	0.350
		1	0.74	0.26	84				
		2	0.64	0.36	184				
		3	0.79	0.21	159				
		4	0.15	0.85	92				
26	Xyl ⁻	(Inoculum	0.56	0.44	92)	53.7	3	< 0.001	0.229
		1	0.70	0.30	139				
		2	1.0	0	110				
		3	0.74	0.26	232				
		4	0.59	0.41	110				
27	Xyl ⁻	(Inoculum	0.56	0.44	92)	48.8	3	< 0.001	0.196
		1	0.75	0.25	261				
		2	0.56	0.44	220				
		3	0.51	0.49	118				
		4	0.42	0.58	132				
29	Xyl ⁺	(Inoculum	0.54	0.46	160)	11.4	3	< 0.01 > 0.001	0.104
		1	0.72	0.38	25				
		2	0.85	0.15	164				
		3	0.87	0.13	197				
		4	0.76	0.24	222				
30	Xyl ⁻	(Inoculum	0.45	0.55	158)	113.4	3	< 0.001	0.355
		1	0.78	0.22	217				
		2	0.56	0.44	94				
		3	0.20	0.80	109				
		4	0.76	0.24	100				
Inoculum composed of mixture of Xyl ⁺ and Xyl ⁻ stock strains		(Inoculum	0.55	0.45	128)	35.3	3	< 0.001	0.208
		1	0.58	0.42	78				
		2	0.70	0.30	66				
		3	0.49	0.51	229				
		4	0.26	0.74	101				

The values of χ^2 and of T (Tschuprow's coefficient of variation) are derived from comparisons between mice in each experiment.

χ^2 and T were computed in the same way as those in Table 2.) This heterogeneity would have been remarkable after a dose of 100 LD 50 if the mice did not differ in resistance (Fig. 2), but might be expected when the mice did differ as in these experiments. Any mouse of above-average resistance would not have received 100 LD 50 in the above control experiment but < 100 LD 50, and so would be likely to yield a sample differing in composition from that of the challenge dose (see introduction).

Seven abscesses obtained from two surviving mice of the original experiment were examined and all contained a preponderance of the Xyl⁺ variant (Table 6).

Table 6. *Mixed-inoculum experiments with Salmonella typhimurium: number of variants found in liver-abscesses of mice surviving inoculation of a mixture of two variants*

In the mixed-inoculum experiment shown in Fig. 3 mice surviving 21 days after inoculation were killed, and any liver abscesses seen were dissected out and cultured.

Mouse	Specimen	No. of colonies	
		Xyl ⁺	Xyl ⁻
a	Abscess 1	> 200	2
	Abscess 2	c. 400	26
	Abscess 3	c. 400	53
b	Abscess 1	c. 50	0
	Abscess 2	c. 150	0
	Abscess 3	c. 150	0
	Abscess 4	c. 120	0

The effect of a terminal breakdown in resistance on the composition of the bacterial population of the heart blood

This experiment was performed to examine the effect of a fatal infection on a concurrent infection by a pathogen of low virulence. Six mice were challenged by intraperitoneal injection with a mixture containing 10^3 of a highly streptomycin-resistant strain of *Salmonella paratyphi-B* ($=10^{-4}$ LD 50 dose) and 10^3 of a virulent streptomycin-sensitive strain of *S. typhimurium* (10^2 – 10^3 LD 50 doses). All the mice died either 3 or 4 days after challenge. Heart blood was removed from each mouse post mortem and plated directly by loop on nutrient agar and on the same medium containing 200 μ g. streptomycin/ml. In each case, a heavy growth was present on the former plates after 18 hr. of incubation at 37° and between ten and seventy colonies were present on the streptomycin agar. Since heavy inocula of this strain of *S. typhimurium* never produced any colonies on streptomycin agar in control experiments, the colonies appearing on these plates were formed by the streptomycin-resistant strain of *S. paratyphi-B*.

DISCUSSION

The slopes of the log-dose/probit-mortality curves of the variants of *Salmonella paratyphi-B* and *S. typhimurium* used in our experiments were 1.81 and 0.66

respectively; this observation is therefore compatible with the result predicted by the hypothesis of independent action, namely, that the slope of such curves will never be greater than 2.0. The slopes also showed that the mice differed in their resistance to *S. typhimurium* and possibly to *S. paratyphi-B*. The LD 50 doses of the variants of each pathogen did not differ significantly. Each variant was represented with about the same frequency in the heart blood removed post mortem from mice challenged with many LD 50 doses of a mixture containing equal parts of all the variants, which showed that there was little difference between their average rates of growth *in vivo*. They were therefore suitable for use in the experiment with mixed challenge doses.

As predicted by the hypothesis of independent action, the compositions of the bacterial populations of the post-mortem heart blood samples differed more and more from each other and from the composition of the challenge dose as the size of the dose became smaller. However, the predictions of this hypothesis were not completely fulfilled for unexpectedly few heart blood samples from mice dying from 1 or < 1 LD 50 dose contained only one variant. Many of these samples yielded an excess of one variant with a small proportion (e.g. 0.2 or less) of the other variant(s). The appearance of the minority was possibly due to a terminal breakdown in resistance caused by the progress of the infection due to the clone descended from only one inoculated bacterium. It is easy to see that such a breakdown might permit the multiplication of other inoculated bacteria, still surviving in the host, to such an extent that they would be present in the heart blood by the time death occurred. This effect was clearly seen when mice were challenged with a lethal dose of *Salmonella typhimurium* (10^2 – 10^3 LD 50 doses) mixed with a non-lethal dose of *S. paratyphi-B* (10^{-4} LD 50 dose). The dose of the latter was so small that it would not have caused either death or bacteraemia if inoculated by itself. However, these bacteria multiplied and appeared in the heart blood of mice fatally infected by *S. typhimurium*.

We hoped next to have been able to distinguish between the hypotheses of complete independence and of partial synergism but the presence of marked differences in host resistance made this impossible in the *Salmonella typhimurium* experiments. It is, however, hard to imagine how synergism could occur between the relatively small numbers of bacteria inoculated in those experiments, and we believe that the hypothesis of complete independence, modified by the occurrence of a terminal breakdown in resistance, provides the most satisfactory explanation for our findings. The same considerations apply to the results of challenge by small doses of *S. paratyphi-B*, but there is some evidence that salmonella do not act independently when the dose contains more than 10^7 bacteria. Maaløe (1948) found that the LD 50 dose of an attenuated strain of *S. typhimurium* was reduced when more than *c.* 10^7 killed organisms of the same species were added to the challenge dose. Under these conditions, the bacteria cannot be acting independently for the presence of one bacterium is clearly influencing the fate of another. For this reason, it is probable that the bacteria only act independently with doses ≤ 2 LD 50 of *S. paratyphi-B* (1.6×10^7 bacteria). In some of our experiments on *S. paratyphi-B*

some mice were given as many as 1.4×10^9 bacteria intraperitoneally; preliminary experiments showed that the LD 50 dose of heat-killed salmonellas given by this route was about 2×10^{10} bacteria, so that even with the largest doses given deaths resulted from infection, not from toxicity.

It must be considered whether the hypothesis of maximum synergism could have accounted for the observed results. This predicts that all the inoculated organisms invariably multiply in each fatally infected host. The simplest assumption to make would be that all these organisms multiply at about the same rate so that the blood at the time of death would always contain the variants in about the same proportions as did the challenge dose. Our results clearly exclude this hypothesis, for the observed values of χ^2 show that the samples from mice dying from small doses differed in composition far more than could be accounted for purely by chance. It might also be argued that when a considerable number of bacteria multiplied, they might invade the blood stream at different times, so that the host would in fact die from the consequences of multiplication of the one bacterium whose progeny had invaded the blood stream before those of other bacteria. Mice which die from small inocula of salmonellas generally die much later than mice which die from large inocula (e.g. in Expt. 2 of Table 2, all mice given 25 LD 50 doses or more died within 24 hr., whereas in mice which died from inocula of 2 LD 50 or less, the median survival time was 6 days). It is possible that the later deaths result from the asynchronous breakdown of localized lesions, each of which may have resulted from the multiplication of only one of the inoculated bacteria; it is to be noted that the abscesses found in survivors appear from their bacterial compositions to have originated in this way. Our data do not exclude this hypothesis but, for reasons given elsewhere, we consider that the hypothesis of independent action is more convincing than its alternatives because it has to be modified by fewer and less restricted assumptions to explain the results of these and of other experiments (see, for example, the discussion of the dose-response curve given below and experiments by Goldberg, Watkins, Dolmatz & Schlamm, 1954).

It has been suggested (Peto, 1953) that the hypothesis of independent action applies generally to infective systems in which an all-or-none response is observed. If this were so, then from the argument presented in the introduction, no infective system would yield a log-dose/probit-mortality curve with slope significantly greater than 2. A search of the literature has shown that this prediction is generally fulfilled since only two curves have been traced with slopes significantly greater than 2 (Parker, Bronson & Green, 1941; Luria & Dulbecco, 1949). In contrast to these results with infective systems, many curves with slopes significantly greater than 2 are reported in the pharmacological literature (Gaddum, 1933; Bliss & Cattell, 1943), showing that synergism occurs between drug molecules.

If the hypothesis of independent action is valid, what type of event would enable one organism to be lethal out of a large number administered? One possibility, that the unique bacterium is a mutant of greater virulence or one with a more rapid rate of growth *in vivo* is excluded in our experiments by

the control tests in which strains recovered from 'one-variant' infections were combined with the original strains of the other variants. It is theoretically possible, but very implausible, that the unique bacterium differs phenotypically from the majority of the bacteria in such a way that it is certain to multiply and cause a fatal infection, while the rest are of such a kind that they will certainly not so multiply. It seems more probable that the inoculated bacteria are more or less the same in both phenotype and genotype and that the outcome of challenge is determined by chance events occurring *in vivo*. The injected bacteria can be considered as being distributed amongst host organs and cells of differing resistance. Each bacterium would have a small independent chance of entering an environment that would permit multiplication and the production of a localized lesion. If each lesion had an independent chance of initiating a fatal bacteraemia, then the observed results would be in accord with the predictions of the hypothesis of independent action.

The experiment using mixtures of variants also tests the validity of the terminal dilution method for the isolation of pure clones of viruses. In this method, hosts are challenged with a virus suspension of such a concentration that P is small. It is assumed that the hosts do not differ in resistance and that the proportion of hosts which responds owing to the growth of one virus particle can be predicted from the Poisson series (Fig. 2). This method will certainly yield pure clones when $p=1$, that is, when the inoculation of one virus particle will invariably produce a response; but when $p < 1$, the validity of the method depends on the applicability of the hypothesis of independent action and on the assumption that a terminal decrease in resistance does not occur.

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The Oxygen Requirement of Growing Cultures of an *Aerobacter* Species Determined by means of the Continuous Culture Technique

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SUMMARY: A formula for calculating the oxygen demand of a growing culture is derived. The fate of substrate-glucose in cultures of *Aerobacter cloacae* was found to depend on the amount of available oxygen and the oxygen demand of the organisms. Anaerobically, cell synthesis and CO₂ production are at their minimum levels and most of the glucose-carbon is converted into ethanol, formic acid, 2:3-butanediol, acetoin and acetic acid. A small supply of oxygen suppresses the formation of ethanol and formic acid but still permits the production of butanediol and acetoin and increases the proportion of glucose-carbon converted to acetic acid, cells and CO₂. A larger supply of oxygen suppresses the formation of butanediol and acetoin and still further increases the yields of cells and CO₂. With an excess of oxygen available, provided the growth rate of the organism is not too near its maximum value, acetic acid production is suppressed and complete conversion of the glucose-carbon into cell and CO₂ occurs. At growth rates very close to the maximum a part of the glucose is converted into acetic acid even with an excess of available oxygen. The metabolism in a batch culture with excess oxygen resembles the metabolism with excess oxygen in a continuous culture in which the organisms are growing at a rate close to the maximum. Means of recognizing and preventing an oxygen deficiency are discussed.

The object of this work was to determine the effect of oxygen supply rate on the fate of glucose carbon in growing cultures of *Aerobacter cloacae*. It is remarkable that although carbon balances with carbohydrate as the carbon source are well defined for anaerobic 'resting' suspensions of the more commonly studied species of bacteria (e.g. see Oginsky & Umbreit, 1954) there is no general agreement about carbon balances under aerobic conditions. Moreover, attention has been largely confined to non-growing or 'resting' organisms and consequently little is known about the carbon balances in cultures of growing organisms. Some organisms under aerobic conditions have been found to convert substrate-carbon mostly to cell-carbon and CO₂ (Clifton, 1946), and Clifton has given approximate equations for the processes. Other workers, e.g. Altermatt, Simpson & Neish (1955), found that substantial amounts of fermentation products, that is products of anaerobic metabolism, were formed from carbohydrate under aerobic conditions. Pasteur (see quotation by Stephenson, 1950) stated that when a yeast was supplied with oxygen the fate of the substrate-carbon depended on the amount of free oxygen which was available. However, no quantitative relationship between the amount of available oxygen in a yeast culture and the carbon balance was established until the work of Maxon & Johnson (1953). The relationship between the amount of available oxygen and the carbon balance in a bacterial culture is investigated in the present paper.

THEORY

Growth rate. For a batch culture in which logarithmic growth occurs, the law of growth is

$$\frac{dx}{dt} = \mu x, \quad (1)$$

where x is the concentration of organisms, t is time and μ is the growth rate constant.

If the culture volume has the constant value V and fresh medium is added at a constant rate F , F/V is known as the dilution rate D . Then in a continuous culture (Monod, 1950; Herbert, Elsworth & Telling, 1956) we have

$$\frac{dx}{dt} = (\mu - D)x. \quad (2)$$

In the steady state $\mu = D$ (since $dx/dt = 0$).

Yield of organism. Monod (1942) showed that in cultures in which the sole growth-limiting factor was the concentration of carbon-substrate the quantity of bacteria produced was a constant fraction, Y , of the quantity of substrate utilized:

$$x = Y(s_0 - s), \quad (3)$$

where s is the concentration of substrate at the time t , s_0 is the initial substrate concentration and $(s_0 - s)$ denotes the amount of substrate utilized. The constant Y is called the yield constant and denotes the amount of bacteria formed from a given amount of substrate utilized.

From (3) we have

$$\frac{dx}{dt} = -Y \frac{ds}{dt}. \quad (4)$$

Oxygen demand of a growing culture. Suppose that for each mole of growth-limiting substrate utilized P moles of oxygen are required. If the concentration of dissolved oxygen is c , the oxygen uptake $(-\Delta c)$ in time t , is given by $-\Delta c = P(s_0 - s)$; hence

$$\frac{-dc}{dt} = \frac{-P ds}{dt}, \quad (5)$$

where $-dc/dt$ denotes the oxygen uptake rate.

From (4) and (5) the oxygen uptake rate is directly proportional to the growth rate $\frac{-dc}{dt} = \frac{P}{Y} \frac{dx}{dt} = \frac{P}{Y} \mu x$. Hence from (3)

$$\frac{-dc}{dt} = P\mu(s_0 - s). \quad (6)$$

For a continuous culture in the steady state we may substitute D for μ ; and generally s is negligible compared with s_0 . Hence (6) becomes

$$\frac{-dc}{dt} \simeq PDs_0. \quad (7)$$

Therefore the oxygen demand of the population of organisms in a continuous culture is directly proportional to the dilution rate and to the concentration of substrate in the fresh medium. P is here called the oxygen demand constant; it may be determined by measuring the substrate utilization and the concomitant oxygen uptake.

Available oxygen in cultures

The amount of available oxygen in a submerged culture depends upon the rate of oxygen transfer from the gas to the liquid phase, that is, the oxygen solution rate (dc/dt); this rate is given by the relationship

$$\frac{dc}{dt} = \phi(c_s - c), \quad (8)$$

where c is the concentration of dissolved oxygen, c_s is the saturation concentration of oxygen and ϕ is a constant which depends on the aeration conditions (Hixson & Gaden, 1950; Bartholomew, Karow, Sfat & Wilhelm, 1950; Chain & Gualandi, 1954; Finn, 1954). One can readily measure the maximum possible oxygen solution rate in an aeration system by means of the sulphite oxidation method of Cooper, Fernstrom & Miller (1944). In the sulphite solution c is virtually zero, in which case $dc/dt \approx \phi c_s$. In this laboratory ϕc_s is given the symbol σ and in this paper it will be called the oxygen absorption coefficient. The oxygen absorption coefficient (σ) is the maximum amount of available oxygen and is expressed as mmole/l./hr. Wise (1950) reported that the polarographic method for determining oxygen absorption coefficients gave a much lower value than does the sulphite method. However, other workers (Bartholomew *et al.* 1950; Chain & Gualandi, 1954) found that the two methods gave results which were in good agreement with each other. Although Wise showed that on theoretical grounds the sulphite method would be expected to give higher values than the polarographic method it is doubtful whether the theory can account for the big differences which Wise found.

Another factor on which the amount of oxygen available to organisms in submerged culture depends is the rate of oxygen transfer from the liquid to the cell. This process, it has been shown for a yeast by Winzler (1941), for some bacteria by Longmuir (1954) and for a mould by Chain & Gualandi (1954), is independent of the concentration of dissolved oxygen unless this is very small as compared with the saturation concentration of oxygen. That being so, it follows from equation (8) that, when the oxygen uptake of the organisms is limited by the concentration of dissolved oxygen, the rate of oxygen uptake must be virtually equal to ϕc_s , the oxygen absorption coefficient (σ). It should be noted, however, that the polarographic method requires quite small population densities of organisms to be used, probably not exceeding 0.1 % dry wt., otherwise the oxygen uptake rate is too rapid to allow of accurate measurement. The conclusions drawn therefore may only be true for population densities of that order.

Rate of substrate catabolism in relation to amount of organism. The rate of substrate catabolism/unit amount of organism will be called the specific

substrate catabolic rate. It is given the symbol K_c when the carbon-substrate is considered. K_c is defined by the relationship

$$\frac{-ds}{dt} = K_c x.$$

From equations (4) and (1) above

$$\frac{-ds}{dt} = \frac{\mu}{Y} x; \quad \text{therefore} \quad K_c = \frac{\mu}{Y}.$$

Summary of symbols and units used: μ = growth-rate constant defined by equation (1), hr.⁻¹; μ_m = maximum value of μ , hr.⁻¹; D = dilution rate, hr.⁻¹; σ = oxygen absorption coefficient, mmole oxygen/l./hr.; P = oxygen demand constant, mole oxygen required/mole substrate-carbon catabolized; Y = cell-yield constant = organism dry wt. produced/unit quantity carbon substrate utilized (for calculating K_c in Table 2 the units are g. organism dry wt./mmole glucose-carbon); K_c = specific glucose catabolic rate, mmole glucose-carbon catabolized/g. dry wt. organism/hr.

METHODS

Apparatus. The continuous culture apparatus which was used and the technique of operating it were as described by Elsworth, Meakin, Pirt & Capell (1956).

Medium A. This medium contained (amounts/l.) = 25.0 g. KH_2PO_4 ; 0.075 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 0.15 g. $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$; 0.20 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.0025 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.005 g. $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.005 g. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; 4.0 g. Na hexametaphosphate (Calgon). Glucose was added in different amounts; 0.33 g. $(\text{NH}_4)_2\text{SO}_4$ was added for each g. glucose. The KH_2PO_4 was adjusted to pH 7.3 with KOH and the pH of the whole medium was 7.2–7.3. The phosphate, ammonium sulphate and glucose were each sterilized separately by autoclaving. The trace minerals were each dissolved separately in water and then mixed with the Calgon solution; a slight pink precipitate (presumably containing some of the manganese) was formed and removed by filtration. Since Calgon is decomposed by autoclaving, the solution of trace elements was sterilized by Seitz filtration.

Medium B. The composition was the same as that of medium A except that ethylenediamine tetracetic acid (1 g./l.) was substituted for the Calgon. The pH value of the chelating agent was adjusted between pH 7 and 8 with NaOH before adding the mineral salts and was re-adjusted to about pH 7 after adding the salts. The stock minerals solution with the complexing agent was sterilized by autoclaving.

Antifoam. Alkaterge C (supplied by Commercial Solvents, Terre Haute, Indiana, U.S.A.) was used for preventing foaming. It was diluted with medicinal paraffin to a strength of 30 % (v/v). Two or three drops of this preparation were added by means of the antifoam meter as soon as the foam formed a continuous layer over the liquid. The concentration of the antifoam preparation was thus maintained near 0.01 % (v/v.).

Temperature. The temperature of the culture was maintained at $37^\circ \pm 0.5^\circ$.

Seed cultures. The organism used was *Aerobacter cloacae* strain NCTC 8197.

(In a preliminary account of this work (Pirt, 1956) the organism was called *Aerobacter aerogenes*.) The inoculum for the reactor consisted of 100–200 ml. of culture produced in shaken flasks. The seed flask was inoculated from a stock culture of the organism on tryptic digest of meat agar. The organism was adapted to the defined medium in two stages. In the first seed flask the concentrations of all salts were decreased to about half strength; in the second seed the concentration of the salts was full strength. In both inocula the glucose concentration was 1 g./l. The oxygen absorption coefficient of the inocula was about 45 mmole/l./hr.

Preparation and preservation of samples. The work required many different analyses to be carried out on the same sample and since they could not be done simultaneously a method of preserving samples was essential. Refrigeration at a temperature just above freezing-point was not adequate. The method adopted was to add one drop of a saturated solution of mercuric chloride to each 50 ml. of sample. This treatment killed all organisms in 0.4 % suspensions and prevented further growth in the cell-free liquor. After removing organisms by centrifugation the samples were stored in the refrigerator (3°). The mercuric chloride did not interfere in any of the analytical techniques; the amount of it was too small to remove any appreciable amount of formic acid.

Dry weight of organisms. The dry weight of organisms used was determined directly by weighing, except for a few cases in which it was calculated from the optical density. A sample containing about 50 mg. dry wt. organisms was centrifuged at 15,000 g. for about 5 min. to separate organisms from medium. The organisms were washed twice by re-suspending in water. The washed organisms, re-suspended in a little water, were then transferred by a Pasteur pipette to a weighed Pyrex evaporating dish. The suspension was evaporated to dryness on a water bath and then dried to constant weight in an oven at 100°–105°. In calculating dry weights from optical densities it was necessary to know the ratio optical density:dry wt. at the particular growth rate which the organisms had, since it has been found, in this laboratory, that this ratio is a function of the growth rate of the organisms.

Cell carbon. Carbon analyses by dry combustion were carried out on samples of organisms grown anaerobically and with excess oxygen. The proportion of carbon in the sample of organisms grown anaerobically was 49.6 %; in that aerobically grown, 50.8 %. The mean of these two values (50.2 %) was used to calculate the amounts of cell-carbon from the dry weight of organisms.

Carbon dioxide. The total CO₂ evolved by the culture is the sum of the CO₂ in the effluent gas and that retained by the medium. The percentage of CO₂ in the effluent gas from the fermentation vessel was determined by means of an Orsat gas-analysis apparatus. The CO₂ retained by the culture medium was determined by the microdiffusion method of Conway (1947); a sample of culture for the purpose was taken anaerobically. This was done by collecting the sample under a layer of liquid paraffin as recommended for blood analysis by Conway. The CO₂ concentration found slightly exceeded the theoretical value calculated by assuming that the dissolved CO₂ was in equilibrium with the CO₂ in the effluent gas.

Total volatile acid. The sample (4 ml.) was acidified with 10 N- H_2SO_4 (0.2 ml.) and steam distilled. Formic acid and acetic acid recoveries were complete after 50 ml. (that is, about 12 vol.) had distilled over. The distilled acid was titrated with CO_2 -free 0.01 N- NaOH , using phenol red as indicator. Sharp end points and very small blank values were obtained without bubbling CO_2 -free nitrogen through the solution to be titrated, provided that it was freshly distilled.

Formic acid and acetic acid. That part of the volatile acid which was destroyed by mercuric salts was taken to be formic acid. A technique for obtaining complete destruction of formic acid was worked out. HgO (310 ± 10 mg.) was placed in a 10 ml. flask, the sample (5 ml.) was added, followed by conc. H_2SO_4 (0.2 ml.), and the contents of the flask were gently boiled under reflux on a sand bath for 20 min. This amount of HgO was more than sufficient to destroy 20 mg. formic acid. On distillation of a portion (4 ml.) of the residual solution, acetic acid was completely recovered and estimated by titration. Acetic acid was identified by the lanthanum nitrate spot test.

Ethanol. The microdiffusion technique of Conway (1947) was used for the determination of ethanol. A correction was made for the small amount of acetoin which diffused with the ethanol.

Acetoin. This substance was estimated colorimetrically by the α -naphthol method described by Neish (1952) with the difference that 1 % α -naphthol was used; this modification decreased the blank value.

2:3-Butanediol. Butanediol was oxidized with bromine water as described by Happold & Spencer (1952). After the reduction of excess bromine with ferrous sulphate, 10 ml. of the solution were half-distilled without fractionation (Neish, 1952). The oxidation products in 4 ml. distillate were estimated by the colorimetric method used for acetoin. Acetoin was subjected to the same procedure to determine the correction to be made for it.

Glucose. The method of ferricyanide reduction was used for the determination of glucose. The reagent was that of Fujita & Iwatake (1931) with increases in the concentrations of constituents to allow a sample of 2-3 mg. glucose to be taken.

Aeration. Oxygen absorption rates were measured by the sulphite method (Cooper *et al.* 1944). The oxygen absorption coefficients found for the various aeration conditions used are given in Table 1.

Table 1. *Oxygen absorption coefficients (σ) and aeration conditions*

σ mmole O_2 /l./hr.	Impeller		Air flow (l./l. culture/min.)
	diameter (mm.)	r.p.m.	
463	73	1030	2.0
228	63	1030	1.0
85	63	1030	0.2
24	63	715	0.2

Note. The air pressure in the reactor was about 6 in. water gauge above atmospheric pressure.



Anaerobic conditions. Anaerobic conditions were obtained by maintaining an atmosphere of nitrogen in the whole apparatus; 0.2 vol. of cylinder nitrogen/vol. culture was sparged through the reactor with stirring at 715 r.p.m. Nitrogen was also bubbled through the medium reservoirs.

Evaporation. Evaporation of the culture was neglected since it was only about 3 ml./l./hr. at 37° with the most intense aeration ($\sigma = 463$) used.

RESULTS

The pH value of cultures. The pH value of continuous cultures in the steady state was 6.7 ± 0.2 with 10.6 g. glucose/l. in the fresh medium. With 19.9 g. glucose/l. the pH value was 6.3 ± 0.2 . In the batch cultures the pH value fell steadily from the initial value 7.3 to 6.6. Automatic pH-control apparatus was not used in this work.

Glucose utilization and maximum cell growth rates at various oxygen supply rates

At both glucose levels used (10.6 and 19.9 g./l.) the addition of more of the medium constituents other than glucose did not affect the population density; growth was limited by the amount of glucose present. The utilization of glucose

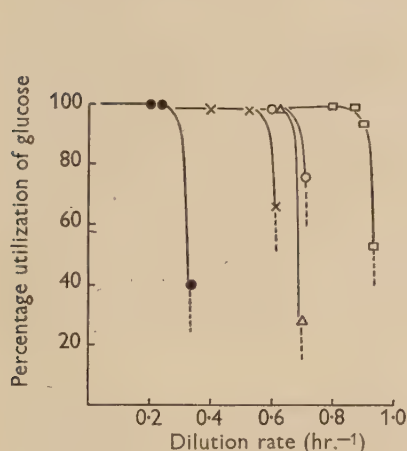


Fig. 1

Fig. 1. Glucose utilization at different dilution rates. Glucose supplied, 10.6 g./l. Oxygen absorption coefficients in mmole/l./hr.: \square , 228 or 463 (medium A); \circ , 228 (medium B); \triangle , 85 (medium B); \times , 24 (medium B); \bullet , 0 (medium B).

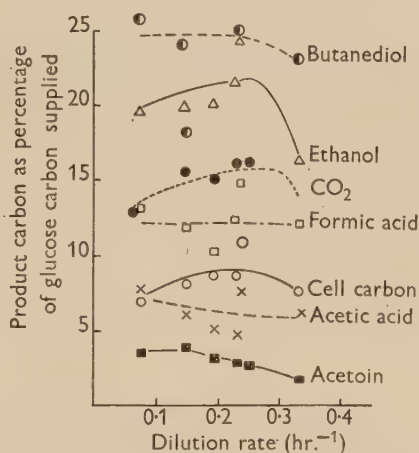


Fig. 2

Fig. 2. End products of anaerobic glucose metabolism as a function of dilution rate in continuous culture with medium B. Glucose supplied, 10.6 g./l.

at different dilution rates with various oxygen absorption coefficients is depicted in Fig. 1. The glucose utilization was virtually complete as long as the dilution rate was not nearly equal to the maximum growth-rate constant (μ_m). The dilution rate at which the glucose utilization decreased sharply was a rough measure of μ_m . It can be seen from Fig. 1 that medium A permitted a higher

growth rate than did medium B. Also from the results with medium B (Fig. 1) it is apparent that when the oxygen absorption coefficient fell below a certain value (between 85 and 228) the maximum growth rate also decreased.

Anaerobic metabolism

When the organism was grown anaerobically the glucose-carbon was accounted for as organisms, CO_2 , acetic acid, formic acid, ethanol, butanediol and acetoin. The average amount of carbon accounted for as known products was 92 %, with a standard deviation of 8. The graphs in Fig. 2 show the percentage of substrate-carbon converted to the various products at different dilution rates. The percentage of total carbon which appeared in the organisms reached a maximum of 9 at a dilution rate of 0.25. At the same dilution rate the percentage of glucose-carbon converted to CO_2 also reached the maximum value of 16 %. The amount of formic acid produced at dilution rates almost up to μ_m was fairly constant, corresponding to about 12 % of the total carbon. About 6 % of the total carbon was converted to acetic acid. Ethanol production under anaerobic conditions accounted for about 22 % of the total glucose-carbon utilized. The slight decrease in ethanol yields at lower dilution rates is attributed to evaporation of ethanol; the decrease in the concentration is roughly what one would expect on the basis of data for ethanol evaporation given by Maxon & Johnson (1953). The butanediol production accounted for about 24 % of the carbon supplied and was uninfluenced by the dilution rate until it was close to μ_m . Acetoin production was relatively small and increased slightly at the lower dilution rates. The sharp decrease in the yields of products at the highest dilution rates was due to the rapid fall in sugar utilization which occurred when the growth rate constant was near its maximum value. The main conclusion to be drawn from the study of the anaerobic metabolism is that it is largely independent of the dilution rate and consequently of the growth rate until, of course, the growth rate is sufficiently near to its maximum value to cause the glucose utilization to be appreciably incomplete.

Fully aerobic metabolism

The metabolism of glucose at 10.6 g./l. with an oxygen absorption coefficient of 228 mmole/l./hr. may next be considered. The average amount of carbon accounted for was 93 %; standard deviation 5. The results are shown in Fig. 3 *a* and *b*. The amount of glucose-carbon converted into cell-carbon reached a maximum corresponding to 55 % of the glucose-carbon supplied, at a dilution rate of 0.6. It was shown with medium B that at dilution rates up to 0.6 practically all of the substrate carbon could be accounted for as organisms and CO_2 . At growth rates near the maximum an appreciable amount of acetic acid was produced. Apart from these products small amounts of ethanol and formic acid (0.3 % of the total carbon, see Fig. 4) were found at the higher flow rates. The yields of organism and CO_2 were, respectively, about 5 and 2.5 times those obtained under anaerobic conditions. At the lower dilution rates lower yields of organism were found and the yield of CO_2 tended to

increase. There were two differences between the results obtained with medium A and medium B. The maximum growth-rate constant was higher with medium A, presumably due to the difference in mineral balance. Secondly, volatile acid production started at a higher growth-rate constant with medium A than with medium B; but in each case the growth-rate constant at which volatile acid production started was close to $0.85 \mu_m$.

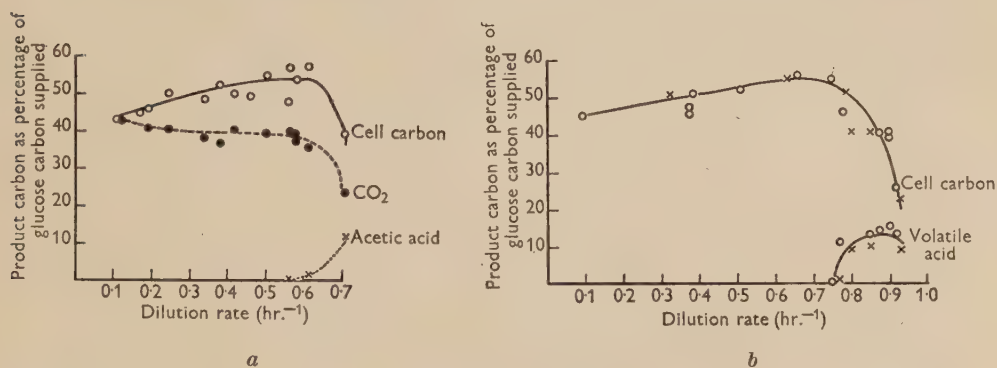


Fig. 3a. End products of glucose metabolism as a function of dilution rate with medium B and an oxygen absorption coefficient of 228 mmole/l./hr. Glucose supplied, 10.6 g./l.

Fig. 3b. End products of glucose metabolism as a function of dilution rate with Medium A. Oxygen absorption coefficients (mmole/l./hr.): ○, 228; ×, 463. Glucose supplied, 10.6 g./l. Volatile acid estimated as acetate. Acetic acid and formic acid were not separately determined in experiments with medium A.

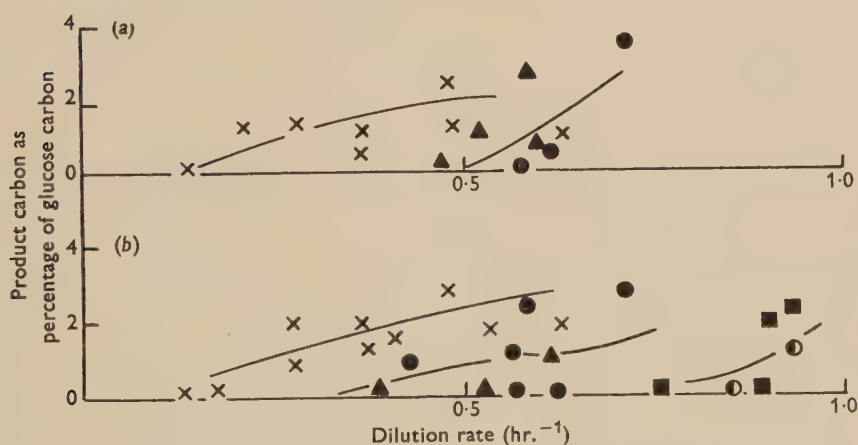


Fig. 4. Ethanol (a) and formic acid production (b) as a function of dilution rate in aerobic cultures. Oxygen absorption coefficients in mmole/l./hr. with medium B: ●, 228; ▲, 85; ×, 24. With medium A: ■, 228; ○, 463. Glucose supplied, 10.6 g./l.

When the oxygen absorption coefficient was increased from 228 to 463 mmole/l./hr. with medium A (see Fig. 3b) the yield of organisms was unchanged and volatile acid production started at the same dilution rate with either degree of aeration. The slightly lower peak conversion of glucose into

volatile acid with the higher oxygen absorption coefficient is probably due to some volatilization of the acid as a result of the intense aeration. Since the increase in the oxygen absorption coefficient caused no apparent change in the end products of glucose metabolism it seems probable that an excess of oxygen was available. We can also decide whether there was an excess of available oxygen by calculating the maximum oxygen demand of the culture by means of equation (7), and comparing it with the maximum amount of available oxygen, that is, the oxygen absorption coefficient. From the results, an approximate value of P for fully aerobic metabolism may be obtained by assuming that the R.Q. is unity and hence equating oxygen uptake with CO_2 production. From the data of Fig. 3a therefore it is concluded that approximately 0.4 mole oxygen is required for each mole of substrate-carbon metabolized. Thus P , the oxygen demand constant, is approximately 0.4 for fully aerobic metabolism. The value of P for growing a yeast under fully aerobic conditions which one can calculate from the data of Maxon & Johnson (1953) is also about 0.4. The calculated maximum oxygen demand of the culture with medium B and 10.6 g. glucose (354 mmole carbon)/l. occurs at a dilution rate of 0.7 hr.^{-1} and is 99 mmole/l./hr. But since the oxygen absorption coefficient was 228 mmole/l./hr. there should have been a large excess of available oxygen.

Partially aerobic metabolism

In order to achieve partially aerobic metabolism with medium B the oxygen absorption coefficient was made less than the theoretical maximum oxygen demand of 99 mmole/l./hr. First, an oxygen absorption coefficient of 85 mmole/l./hr. was used and secondly an oxygen absorption coefficient of 24 mmole/l./hr. With these oxygen absorption coefficients one calculates from equation (7), taking $P=0.4$, that the oxygen solution rate should become a limiting factor at dilution rates of 0.6 and 0.2, respectively.

The results obtained using an oxygen absorption coefficient of 85 mmole/l./hr. are shown in Fig. 5. With dilution rates less than 0.45 the metabolism was the same as that with an oxygen absorption coefficient of 228. It may be concluded, therefore, that the metabolism was fully aerobic at dilution rates up to 0.45. At higher dilution rates the yields of organism and CO_2 fell below those found with an oxygen absorption coefficient of 228. Also, at a dilution rate of 0.45 acetic acid production started, whereas with an oxygen absorption coefficient of 228 acetic acid production did not occur until the dilution rate exceeded 0.6. Acetoin or butanediol were not formed until the dilution rate reached 0.6 when traces (about 0.5 % of the total carbon) were detected. Traces of formic acid and ethanol were produced as the graphs in Fig. 4 show. The average amount of glucose-carbon accounted for was 90 %; standard deviation 5. The dilution rate of 0.45 at which the carbon balance began to diverge from that characteristic of fully aerobic metabolism is in reasonably good agreement with the calculated dilution rate (0.6) at which the oxygen solution rate should become a limiting factor.

The graphs showing the results obtained with an oxygen absorption coeffi-

cient of 24 mmole/l./hr. are given in Fig. 6. The average amount of carbon accounted for was 87%; standard deviation 6. Under these conditions, at dilution rates up to 0.2, the end products found (i.e. organisms and CO_2 only) were the same as with higher oxygen absorption coefficients. The metabolism

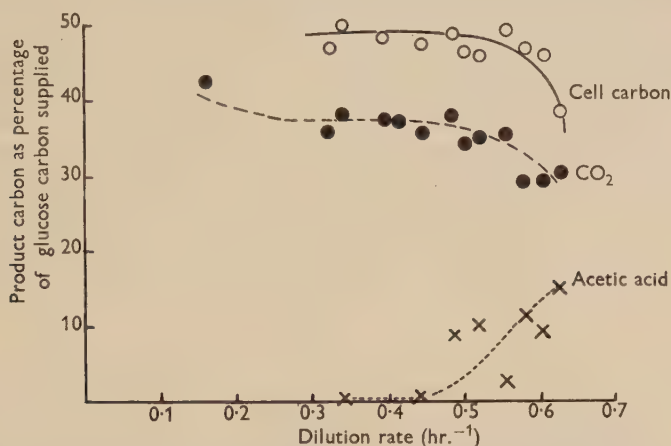


Fig. 5. End products of glucose metabolism as a function of dilution rate with an oxygen absorption coefficient of 85 mmole/l./hr. Medium B; glucose supplied, 10.6 g/l.

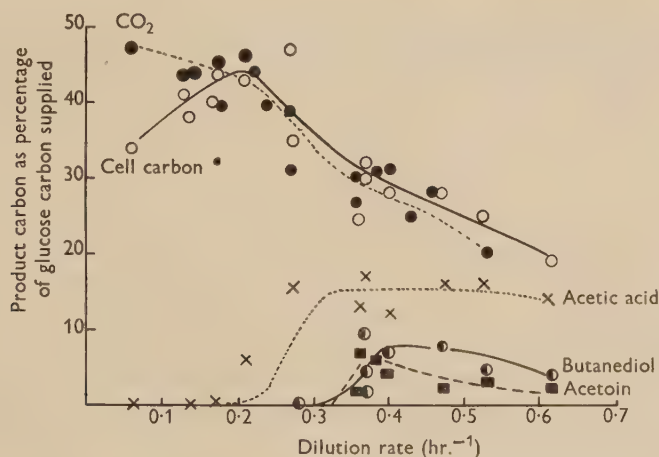


Fig. 6. End products of glucose metabolism as a function of dilution rate with an oxygen absorption coefficient of 24 mmole/l./hr. Medium B; glucose supplied, 10.6 g/l.

must therefore be fully aerobic at dilution rates up to 0.2, which is the same value as the calculated dilution rate at which the oxygen solution rate should become limiting. When the dilution rate exceeded 0.2 acetic acid was the first new product to appear. Acetic acid production reached its maximum at a dilution rate of 0.3. At this dilution rate (i.e. when acetic acid production reached its peak) acetoin and butanediol production began. Formic acid and ethanol were produced in small quantities (1-3% of the total carbon) at the

higher dilution rates (Fig. 4). Apparently formic acid and ethanol were formed in appreciable amounts only under almost anaerobic conditions. The fact that aeration suppressed ethanol formation but permitted butanediol production was also observed in studies of 2:3-butanediol production by *Aerobacter aerogenes* (Ledingham & Neish, 1954). It is concluded therefore that in partially aerobic metabolism the nature of the end products of carbon metabolism depends on the oxygen deficiency, which is defined as the difference between the amount of oxygen available and the amount required for fully aerobic growth. A small oxygen deficiency will lead to the formation of acetic acid, a larger one causes butanediol and acetoin production; and with no oxygen available formic acid and ethanol production occur and acetic acid production is decreased. Presumably the oxygen deficiency exerts its effect by governing the concentration of dissolved oxygen. The pH value also affects the ratio of the amounts of different products formed with partially aerobic metabolism.

Tests for other metabolic products

Besides those substances which this strain of *Aerobacter cloacae* produces a number of other likely products were tested for but found to be absent. They were: pyruvic acid in partially aerobic and anaerobic metabolism; diacetyl in partially aerobic metabolism; lactic acid with fully aerobic, partially aerobic and anaerobic metabolism; succinic acid with fully aerobic metabolism. Hydrogen, which may be produced by the dehydrogenation of formic acid, was not tested for. Some determinations of the total non-volatile carbon in the cultures (by dry combustion) revealed that 97–100 % was accounted for as known products; hence most of the glucose-carbon not accounted for as known products was probably in the volatile fraction.

Metabolism in batch culture

The growth-rate constants of batch cultures started from seed cultures varied from one culture to another and generally were lower than the maximum growth-rate constant always obtained in continuous cultures. Presumably this was because better adaptation to the new medium occurred when the culture was prolonged. The formation of metabolic products during growth of the organism in batch culture with medium B is depicted in Fig. 7. The glucose concentration was *c.* 10 g./l. and the oxygen absorption coefficient 228 mmole/l./hr. The CO₂ produced was not measured but probably accounted for at least 30 % of the total carbon (from measurements of CO₂ production in continuous cultures with the growth-rate constant very close to its maximum value). Assuming a value of 30 % for the CO₂ production the carbon recovery would be 91 %. The results show that acetic acid was produced throughout the batch culture and accounted for 15 % of the glucose-carbon utilized. A small amount of formic acid was produced towards the end of the culture but it was metabolized after the glucose was exhausted. No appreciable amounts of ethanol, acetoin or butanediol were detected. In another batch culture with a higher oxygen absorption coefficient (463 mmole/l./hr.) and using medium A

the growth-rate constant was higher but organisms and acetic acid were produced in the same proportions as the data in Fig. 7 indicate. The cell-yield constant (Y) in the batch cultures was 0.32 g. dry wt. organisms/g. glucose, which is much lower than the maximum (0.44) obtained in a continuous culture with a dilution rate of 0.6 and an oxygen absorption coefficient of 228, but about the same as the yield obtained in a continuous culture operating at the highest growth rate. The possibility that the acetic acid was metabolized

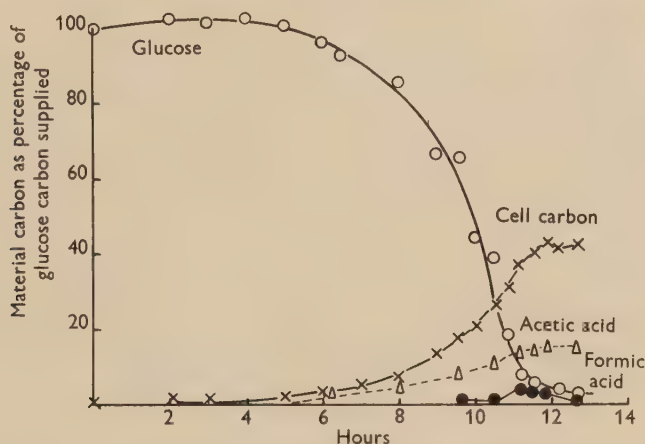


Fig. 7. Products of glucose metabolism in batch culture as a function of time. Medium B; glucose supplied, 9.6 g./l.; oxygen absorption coefficient 228 mmole/l./hr.

later in the batch culture after exhaustion of the glucose was not investigated. Thus the metabolism in these batch cultures seems the same as fully aerobic metabolism in a continuous culture with the growth rate of the organism very close to its maximum value. In view of the decreased CO_2 yields at growth rates close to the maximum, the value of the oxygen demand constant in batch cultures probably was less than 0.4, however, using the value 0.4 for P one calculates by means of equation (6) that the maximum oxygen demands in these batch cultures were 104 mmole/l./hr. with medium A ($\mu=0.78$) and 61 with B ($\mu=0.46$). Hence with oxygen absorption coefficients of 463 and 228 mmole/l./hr., respectively, oxygen should have been present in large excess throughout the growth periods.

Effect of increasing glucose concentration

The results so far described concern the metabolism of glucose at a concentration of 1% (w/v). An investigation of the metabolism using 2% (w/v) glucose media was also made to see whether the metabolism would be as one would predict from the study of the lower strength media. A complication arose because, with the utilization of greater amounts of NH_3 , the steady state pH value (6.3) was lower than that with 1% glucose (pH 6.7); at the moment one cannot say whether this affected the results. The oxygen absorption coefficient was 228 mmole/l./hr. The glucose utilization over the range of

dilution rates studied was practically 100 %; the results are shown in Fig. 8. The average amount of carbon accounted for as known products was 88 %; standard deviation 7. At dilution rates below 0.4 the only products found were organisms and CO_2 , and the number of moles of CO_2 produced/mole glucose-carbon was 0.4, as with 1 % glucose. Hence it is concluded that the metabolism is fully aerobic with 2 % glucose and an oxygen absorption coefficient of 228 mmole/l./hr. at dilution rates below 0.4. When the dilution rate

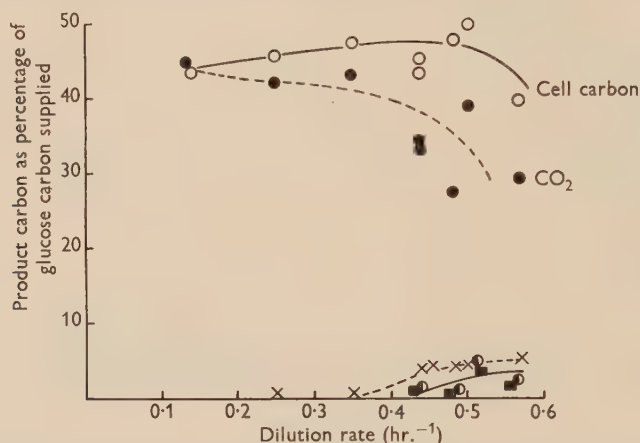


Fig. 8. Products of glucose metabolism as a function of dilution rate with a glucose supply of 19.9 g./l., medium B and an oxygen absorption coefficient of 228 mmole/l./hr. Acetic acid, \times ; acetoin, \blacksquare ; butanediol, \bullet .

exceeded 0.4 acetic acid was produced and butanediol and acetoin appeared at a slightly higher dilution rate. Small amounts of formic acid and ethanol (1–2 % of the total carbon) were formed at the higher dilution rates. At dilution rates in excess of 0.4, therefore, the metabolism was partially aerobic. The value of the oxygen demand constant calculated from the CO_2 production for fully aerobic metabolism with glucose at the 2 % level is close to 0.4 as it is with 1 % glucose. With an oxygen absorption coefficient of 228 mmole/l./hr. and a glucose concentration of 19.9 g./l. (666 mmole carbon) one calculates that the oxygen solution rate should not become a limiting factor until the dilution rate exceeds 0.86. There was, therefore, a considerable discrepancy between the theoretical value (0.86) and the value found (0.4) for the dilution rate at which the metabolism changed from the fully to the partially aerobic type. This discrepancy is in contrast to the much better agreement between the calculated and the values found for the dilution rate at which the change from fully aerobic to partially aerobic metabolism occurred with 1 % glucose. To account for the discrepancy it was thought that the oxygen absorption coefficient might decrease with increase in the concentration of organisms as it does with moulds (Chain & Gualandi, 1954). It was found, however, that freeze-dried *Aerobacter cloacae* added at a concentration of 0.8 % (w/v) to a sulphite solution decreased the oxygen absorption coefficient by only 5–10 %

(the measurements were carried out quickly with 0.001 M-CuSO₄ as catalyst). Hence diffusion of oxygen from the gas to the liquid seemed to be little affected by bacteria concentrations up to 0.8 % dry wt. It seems unlikely therefore that the occurrence of oxygen-limited metabolism with the 2 % glucose medium and an oxygen absorption coefficient of 228 mmole/l./hr. was because the oxygen solution rate was a limiting factor.

DISCUSSION

Maximum oxygen uptake rate with limited aeration

This work shows that at bacterial population densities equivalent to *c.* 0.4 % dry wt., when aeration becomes limiting the oxygen uptake rate is roughly equal to the maximum oxygen solution rate (σ) as determined by the sulphite method. When, however, the population density is equivalent to 0.8 % dry wt. aeration becomes limiting when the oxygen uptake rate is considerably less than σ . This suggests that at low population densities oxygen solution rate is the sole factor limiting the availability of oxygen to the bacteria but at high population densities some other factor also is concerned. This finding is to be contrasted with that of Maxon & Johnson (1953) who concluded that in yeast cultures with population densities up to the equivalent of 4 % dry wt., aeration is never limiting until the oxygen uptake rate is equal to σ .

Recognition of oxygen deficiency

With the strain of *Aerobacter cloacae* used here and the pH value between 6 and 7 the production of acetic acid may be the first sign that there is an oxygen deficiency. With increasing oxygen deficiency 2:3-butanediol and acetoin appear in the medium and finally, as the conditions become nearly anaerobic, ethanol and formic acid appear in large amount. Decreased yields of organisms and CO₂ also indicate an oxygen deficiency. Acetic acid is also produced when there is an excess of available oxygen if the organism is growing at or near to its maximum rate. This effect is analogous to one which Maxon & Johnson (1953) observed to occur in yeast metabolism. They showed that a yeast with excess oxygen completely converted glucose to cell-carbon and CO₂ provided the growth rate was not too high; however, when the yeast growth rate exceeded a certain critical value then ethanol was produced even with excess oxygen. To account for ethanol production by yeast in the presence of excess oxygen Maxon & Johnson (1953) suggested that when a certain rate of glucose catabolism/g. organism is reached, 'the oxidative enzymes of the yeast have been saturated and the glycolytic enzymes which are present in greater abundance, operate to supply additional energy for growth'. The rate of glucose catabolism/g. organism (K_c) for carbon-substrate-limited growth is shown at the beginning of this paper to be the quotient μ/Y . The K_c values for *A. cloacae* under certain critical conditions are given in Table 2, from which it is clear that under conditions of oxygen deficiency the organisms can utilize glucose more rapidly than they can in the presence of oxygen. The critical K_c

values above which acetic acid formation occurred in the presence of excess oxygen were 48 mmole carbon/hr./g. dry wt. when the glucose consumption was 10 g./l. and 31 mmole/hr./g. dry wt. when the glucose consumption was 20 g./l. The lower critical value of K_c when the glucose consumption was 20 g./l. suggests that the occurrence of partially aerobic metabolism in that case was not due to 'saturation' of the enzymic pathway to free oxygen.

Table 2. *Specific glucose-carbon catabolic rates of Aerobacter cloacae under some critical conditions*

Conditions*	Growth-rate constant (hr. ⁻¹)	Specific carbon catabolic rate (K_c) mmole carbon/hr./g. dry wt. organism
With excess oxygen at growth rate at which acetic acid production began	0.60	48
At maximum growth rate with excess oxygen	0.72	65
Partially aerobic metabolism (σ , 24), close to maximum growth rate	0.50	91
Anaerobic metabolism close to maximum growth rate	0.25	105

* These results were obtained using medium B with 10.6 g. glucose/l.

Prevention of oxygen deficiency

To show that aeration is not a limiting factor in an aerobic process it is not sufficient to demonstrate that the process is unaffected by increasing the rate of air flow through the culture as some recent papers suggest. Increasing the air flow rate does not necessarily increase the oxygen solution rate and consequently the amount of available oxygen. To demonstrate an increase in oxygen available to the culture one must demonstrate that the oxygen absorption coefficient is increased. Once the oxygen absorption coefficient is fixed, to ensure that an excess of oxygen is available to the organisms the oxygen demand must be made less than the oxygen absorption coefficient. From equation (6) it follows that the oxygen demand can be decreased either by lowering the growth-rate constant or the amount of substrate supplied. For example, applying the results to shake-flask culture in which the oxygen absorption coefficient is 40 mmole/l./hr. (a fairly high value for shake-flasks; see, for example, Finn, 1954) we find that when the generation time of the organism is one hour (i.e. $\mu=0.693$) and P is 0.4 the glucose concentration must not exceed 4.33 g./l. if excess oxygen is required in the medium throughout the growth of the culture.

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The Nuclear Cycle of *Myxococcus fulvus*

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SUMMARY: The life cycle of *Myxococcus fulvus* consists of the following stages: germinating microcysts, young vegetative cells, intermediate cells, mature cells and resting microcysts. Germination takes place by simple elongation, or germ-tube formation. The nucleus of the germinating microcyst is a single oval or elongate body. The young vegetative cell should probably be referred to as multinucleate because the nucleus consists of a string of lobate bodies. In intermediate and mature cells the nucleus is either bi-lobed or oval, with the presence of entire edges. Details of the electron micrographs suggest a nuclear membrane. Microcysts normally have a single nucleus, and possibly possess nucleoli. Resting microcysts consist of a thin outer shell, a thick inner shell, cytoplasm and a nucleus.

In studying the cytology of *Myxococcus virescens* Badian (1930) presented evidence of the presence of discrete chromatinic bodies, interpreted as nuclei, in vegetative cells of this myxobacterium. Beebe (1941), studying the cytology of *M. xanthus*, reported the presence of nuclear structures clearly analogous to those observed by Badian (1930). Complex nuclear cycles were postulated by Badian and by Beebe. These hardly seem justified, however, on the basis of the evidence presented. Three species of terrestrial myxobacteria were studied by Klieneberger-Nobel (1947) who used mainly the acid Giemsa method of Robinow (1944). These included *M. fulvus*, *M. virescens* and *Chondroccoccus exiguus*. Young vegetative cells were reported to contain two transverse nuclear structures which divided lengthwise into four before cell-division occurred. When a culture became well established, a new development occurred in some of the cells. The two nuclear structures in each cell increased in size but did not divide. Each cell now shortened and became round with the simultaneous fusion of the two nuclear elements first into a rod-like and eventually into a round chromatinic body. This cell was called a 'fusion cell'. According to Klieneberger-Nobel, the fusion cell now enlarged and stretched to an oval form. At the same time the nuclear body of the fusion cell elongated into a rod or dumbbell-like structure which divided into two (or four) nuclear elements. Subsequently, the cell again rounded up and became smaller, developing into a microcyst with a tough dense outer layer.

In this laboratory it was found that the nucleus of *Myxococcus fulvus* could often be demonstrated with the electron microscope in whole untreated cells. The present investigation is a study of the nuclear cycle in *M. fulvus* using light and electron microscopy.

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METHODS

Myxococcus fulvus, University of Washington strain M6, was used in this investigation. This strain was selected for cytological study, from a number of well-defined strains of *M. fulvus* isolated in this laboratory, for several reasons. Slime was formed in relatively small amounts as compared to most strains, and hence there was little interference with the fixation and staining of habit patterns. Furthermore, strain M6 was able to form fruiting bodies when the amount of nutrient material was decreased to a very low concentration. Under these circumstances the fruiting bodies were so small that they could be removed intact by the agar block technique.

A dung decoction agar medium was used for the cytological studies. This medium was prepared in the following manner. A dung extract was prepared by boiling 1 vol. of rabbit dung with 2 vol. of water for 10 min. The supernatant fluid was removed, autoclaved and added in a concentration of 5 % (w/v) to sterile 1.5 % (w/v) water agar. To this was added 1 % (w/v) of a heavy suspension of heat-killed bacteria consisting of equal portions of *Escherichia coli* and *Proteus vulgaris*. Stock cultures of *Myxococcus fulvus* were maintained on 1 % water agar overlaid with a heavy suspension of autoclaved *E. coli*.

For the study of nuclear structures by light microscopy, impression films were prepared by the OsO_4 -Giemsa method of Robinow (1947). Entire colonies or any portion thereof could be stained in this manner.

For electron microscopy *Myxococcus fulvus* was grown on collodion or formvar films. Collodion films were prepared directly on the surface of the dung decoction agar medium following the method of Hillier, Knaysi & Baker (1948). Small squares were cut from the agar at the desired location in the colony and the film floated off in water. Some of the preparations were fixed by exposure to OsO_4 vapours for 2 or 3 min. The formvar films were prepared in the same manner as the collodion films, except that chilled Ringer's solution was used instead of distilled water to cover the agar surface. The subsequent treatment was the same as with collodion film.

Material for ultra-thin sections was prepared according to the method of Chapman & Hillier (1953). The sections were cut on a Spencer rotary microtome equipped to cut thin sections by thermal expansion.

Electron microscopy was performed with the RCA electron microscope Model EMU-2B. Photomicrographs were taken with a Leitz microscope with a combination of a $\times 92$, 2 mm. apochromatic objective and a $\times 15$ compensating ocular and a Bausch and Lomb type R camera. Eastman M plates were used.

RESULTS

The myxobacterial colony

The creeping motility of terrestrial fruiting myxobacteria determines the morphology of the colony. The vegetative cells, instead of piling up as do most eubacteria, move outward over the surface of the agar, forming a very thin film which is often only one layer of cells in thickness. Fruiting-body production

first begins at or near the point of inoculation and then extends outward in the colony, the fruiting bodies usually appearing in concentric rings around the central portion of the colony.

Three regions were found in the colony of *Myxococcus fulvus*. These three regions are evident by macroscopic appearance, and the cells in these regions are so characteristic that one can tell from which region they came by examination of the nuclear material. The first region is at the periphery of the colony and contains rapidly proliferating vegetative cells which have access to fresh nutriment. This region is very narrow, even in large colonies not more than about 1–2 mm. wide. It has more cells/unit area than any region except for the fruiting body. Because of this, it has a delicate raised appearance.

The second region lies behind the first region, in which active multiplication of cells is occurring. Radial ridges and concentric rings of slime are characteristically observed in this region. This may be the result of the secretion of slime by the generations of passing cells and the grouping of the cells left behind. The cells in the second region, however, are present for the most part as a monolayer. The most typical cells are long and slender, and on drying shows a marked constriction near the middle. The cells in this region are called intermediate cells because of their age and position in the colony.

The third region is the area of fruiting body production and represents the oldest part of the colony. As the cells age they gather to form the fruiting bodies. With the passing of time the intermediate areas are practically cleared of cells except for areas of fruiting. The concentric rings now become concentric rings of fruiting bodies. Microcysts can be found abundantly within the fruiting bodies and in some cases adjacent to the fruiting bodies.

The life cycle

Germination. Mature microcysts of *Myxococcus fulvus*, strain M6, remained undifferentiated when stained by the Robinow-Giemsa method. When placed on nutrient medium, they began to germinate in *c.* 3 hr. and at this time showed a single chromatinic structure which was usually round and excentric though sometimes centrally located. As the cells began to elongate, the chromatinic body, or nucleus, most frequently appeared as a transverse band. In some cases the elongation took the form of a narrow tube, which in some individuals resembled the germination tube described in some yeasts. It is of interest to note that Kunicki-Goldfinger (1949) observed the formation of germ tubes during the germination of microcysts from a species of *Sporocytophaga*. As the cell continued to elongate, the transverse chromatin band appeared to rotate to a longitudinal position. When a cell produced a germ tube, the chromatinic body often appeared at the junction, distributed between the two portions. Some cells resembled a bowling pin with the chromatinic body at the neck. As the cells elongated further, the nuclear material divided into an irregular number of masses as indicated by nuclear stains (Pl. 1, fig. 1).

When germinating microcysts were stained with Sudan black in ethylene-glycol, granules taking the Sudan stain were normally found at or near one or both ends of the elongating cells. When the column of the electron microscope

was evacuated, holes often developed in the regions of the cells corresponding to those which stained with Sudan black. The holes became more obvious when the preparations were shadowed and, as shown in Pl. 1, fig. 2, the cells were often torn and distorted. Holes did not appear in preparations of germinating microcysts fixed with OsO_4 . In view of the staining behaviour with Sudan black and volatilization in the electron microscope, it seems likely that the granules are lipid in character.

Nuclei were not demonstrable in preparations of germinating microcysts under the electron microscope. However, small electron-dense granules were often found in cells of lesser opacity.

Young vegetative cells. The swarm cell of the advancing front in the first region of the colony had a characteristic appearance. With development of the germinating microcyst into the vegetative cell the lipid-like droplets or granules diminished in size and disappeared. Giemsa stains of swarm cells are shown in Pl. 1, fig. 3. The ends of the cells were devoid of chromatinic material. The central portion of the cell was quite well filled by a number of irregular masses of chromatin, apparently attached to each other by chromatinic threads. In electron micrographs, cells from the advancing front were differentiated into relatively transparent and opaque areas (cf. Pl. 1, fig. 4). The ends of the cells were quite dense, representing the cytoplasm. More of this dense formless material, which must also be cytoplasm, was scattered throughout the cells.

Intermediate cells. The intermediate cells of *Myxococcus fulvus* left behind by the advancing frontal wave were long and slender. A Giemsa preparation of such cells is shown in Pl. 2, fig. 5. The clear cytoplasmic areas at each end were longer than in the younger cells, and the edges of the nuclei are entire. The nucleus was long and filled the entire width of the cell. There was sometimes a constriction in the cell membrane near the centre of the cell, and the bi-lobed nucleus, which occupied a central position, also had the constriction. The constriction of the membrane may be an artefact produced by drying since it is not visible in the living cells. These may be the cells which Klieneberger-Nobel (1947) called 'fusion cells', because she considered that two nuclei were fusing. Since there is at present no method of demonstrating multi-cellularity in myxobacteria, it was not possible to determine whether this cell was dividing or whether the nuclear material was fusing.

When the cells of the intermediate region were studied by electron microscopy, it was found that the densities of the nuclear and cytoplasmic material were sometimes reversed, though the two types never appeared in the same preparation. The most frequent form was the one in which the cytoplasm appeared more dense than the nucleus; such cells are shown in Pl. 2, fig. 6, and Pl. 2, fig. 7. On some occasions the reverse was true, and the nucleus appeared quite dense; Pl. 2, fig. 8, shows this type of cell. Small dense granules, apparently intranuclear, were sometimes observed (cf. Pl. 2, fig. 7). Similar granules were observed at other stages of development, and these are hereafter referred to as organelles.

Mature cells. As judged by light microscopy, the mature cells of *Myxococcus fulvus*, just before encystment, were much shortened and appeared to have

a single nucleus, centrally located, which occupied a major portion of the cell. Giemsa stains of mature cells are shown in Pl. 3, fig. 9; electron micrographs in Pl. 3, figs. 10 and 11. The stained cells looked relatively uniform. In the Giemsa preparations the nucleus was oval and the cytoplasm seemed to shrink, possibly because of hydrolysis. This left the impression that the nucleus had a larger diameter than the rest of the cell, but this may have been due to halation.

The electron micrographs of mature cells showed much more variation. The less dense nuclei (Pl. 3, fig. 11) were seen most frequently. The cells were shortened and the nucleus appeared as a single structure. In many cases small dense granules or organelles were again seen.

The more dense nuclei were less common and are shown in Pl. 3, fig. 10. The presence of organelles was very striking. As a general rule, these structures were confined to the nucleus. In some instances they appeared to be extruded from the nucleus or from the cell. The fact that they appeared in both the dense and light nuclei, lent support to the belief that the light and dark nuclei were indeed identical structures.

When the cells rounded up, they appeared by the Giemsa stain to be almost entirely nuclear material. The oval cells in Pl. 3, fig. 9, are comparable to the shortened broad cell in Pl. 3, fig. 10. The nucleus was found along one side. It seems likely that when hydrolysed and impregnated with stain, the nearly round cells (Pl. 3, figs. 11, 12) would appear entirely stained since the narrow shrunken cytoplasm would doubtless be beyond the resolution of the light microscope. When cells of this type were shadowed they appeared flattened (Pl. 4, fig. 13). The mature microcyst was found to be a sphere, not subject to flattening, or differential staining, and casting a long oval shadow (Pl. 4, fig. 14).

As with other organisms, old cultures of myxobacteria have 'involution forms'. Although these cells appear in some of the photographs no effort is made to describe them.

Microcysts. Intact normal microcysts revealed no internal structure when studied by ordinary hydrolysis followed by staining, or by electron microscopy. By subjecting microcysts to disintegration and by the study of ultra-thin sections, evidence was obtained that microcysts consist of a thin outer shell or coat, a thick inner shell, cytoplasm and nucleus.

Suspensions of microcysts were placed in the Mickle disintegrator for a total period of 30 min.; at 5 min. intervals samples were removed, fixed with OsO_4 and examined with the electron microscope. One cell in Pl. 4, fig. 15, shows the outer shell cracked but still encircling the core. A cell with an intact inner shell is shown in the same figure. Free outer shells, shadowed with nickel (Pl. 4, fig. 16) had much the same appearance as cell walls of eubacteria. When the inner shell was ruptured, as shown in Pl. 4, fig. 17, it appeared to have a firm consistency as suggested by the appearance of torn edges. In some of the cells shown in Pl. 4, fig. 17, the material on the inside of the inner shell had evidently been extruded and disintegrated.

Ultra-thin sections of microcysts are shown in Pl. 5, figs. 18 and 19. During manipulation, it was not uncommon for the outer shell to become detached

from the sectioned microcysts; hence this structure was not clearly apparent in all cells. The inner shell was very opaque and in most cases clearly distinguishable. As in germinating microcysts, there was evidence of the occurrence of lipid within or below the second shell.

A soft extrudable core was found inside the inner shell. Since no stains were made of the ultra-thin sections, their staining properties are not known. From studies on germination, however, it is logical to assume that the less dense material is nuclear in nature. This is in keeping with the density relationships observed in most cells in which relatively transparent nuclei and opaque cytoplasm were found. As in intermediate cells and mature cells, organelles were present which appear to be intranuclear.

DISCUSSION

In the typical vegetative or swarm cell the nuclear bodies increase to 2, 3, 4 or more masses. They are irregular in number and shape and appear to be connected by chromatinic threads. The polar areas are normally devoid of nuclear material. The electron micrographs confirm this picture. The cytoplasmic polar caps are entirely dark, and scattered throughout the central area is more dense material which must also be cytoplasmic in nature. The light areas, interspacing the dense material, appear as lobate masses, connected in a string and often lying on one side of the cell. These findings are much like those obtained by Chapman & Hillier (1953) in ultra-thin sections of vegetative *Bacillus cereus* in which the nuclei consisted of a series of swollen structures connected with constricted portions of the same material which were reported to be 'not necessarily axial'.

A number of investigators have attached importance to the number of chromatinic bodies which appear in bacteria (Robinow, 1947; Klieneberger-Nobel, 1945; Bisset, 1950; Grace, 1951; Fitz-James, 1954). Klieneberger-Nobel (1947) emphasized the occurrence of two to four chromatinic bodies in cells in preparations of young myxobacteria. Fitz-James (1954) followed the changes in the chromatinic bodies of *Bacillus cereus* from the resting cell to the first division and claimed to have found a constant number which duplicated. The general impression with *Myxococcus fulvus*, however, is that there is great irregularity in the number of chromatinic bodies.

The transparency of the bacterial nucleus to the electron beam has been a controversial issue. It has been reported to be more transparent than the cytoplasm (Cosslett, 1948; Robinow & Cosslett, 1948; Hillier, Mudd & Smith, 1949), or to consist of a dense matrix (Piekarski, 1939; Knaysi & Mudd, 1943; Knaysi, Hillier & Fabricant, 1950). The density of the nucleus reported here cannot be attributed to or correlated with the treatment to which the organism has been subjected. The dark nuclei were found in the cells grown on collodion and formvar films, and directly on dung agar, but never with fixed specimens. Light nuclei appeared in unfixed cells grown on collodion and formvar films and on cells grown on formvar film and fixed with OsO_4 . The cells were all grown on dung agar, and various lots of dung agar, even though made by the

same formula, may vary. Knaysi (1951) showed that the omission of a nitrogen supply caused an increase in the transparency of the cytoplasm in *Bacillus megaterium*. It is difficult to believe, however, that the nitrogen supply could vary to this extent with the dung agar medium.

Small dense bodies, or organelles, were found at all stages of development of *Myxococcus fulvus* except during the period of active multiplication. These organelles appeared to be primarily intranuclear. While their nature is not known, it is tempting to consider that they are nucleoli. To the best of our knowledge, with one possible exception (Knaysi *et al.* 1950), structures exactly like the organelles shown in Pl. 1, fig. 2; Pl. 2, fig. 7; Pl. 3, figs. 10 and 12 have not been described in other bacteria. Large electron-dense bodies were found by Knaysi *et al.* (1950) in an avian strain of *Mycobacterium*. These were located chiefly near the ends of the cells and were considered to be nuclei. Small nearly spherical dense structures were found, however, by these investigators in transparent bodies which were considered to be cell sap vacuoles. Electron-dense bodies found in mycobacteria by Winterscheid & Mudd (1953) were identified as mitochondria. Nuclei were considered to be less dense than the cytoplasm and to be located between the electron dense bodies. Structures analogous to the cell sap vacuoles were not resolved.

Brieger, Cosslett & Glauert (1954) studied the reproductive changes in strains of avian tubercle bacilli and concluded that the large electron-dense bodies were not nuclei. These investigators reported the formation of discrete intracellular units inside elongating filaments of tubercle bacilli. Subsequently massive production of small rods occurred. Although details of the transformation from filaments to short rods were not observed, it was suggested that processes such as division by retraction or liberation of intracellular units must be considered in addition to transverse fission. Transparent bodies, referred to as vacuolized structures, each with a limiting membrane, were observed in many preparations, and it was noted that they had a location similar to that of the intracellular units. The question was raised as to whether the intracellular units, which developed into rods, and the transparent bodies were independent structures or were the same structures in different stages of development. No mention was made of the occurrence of small dense granules within the transparent structures as observed by Knaysi *et al.* (1950). Further evidence against the hypothesis that the large electron-dense bodies are nuclei was provided by Glauert & Brieger (1955) in a study on *Mycobacterium phlei*. The dense bodies were found to contain metaphosphates and to show metachromasia. The possibility that they might be mitochondria was discounted. According to Glauert & Brieger (1955) there is still uncertainty as to the location of the nucleus in mycobacteria. If the large electron-dense bodies are not nuclei, is it possible that the structures referred to as cell sap vacuoles or transparent bodies are nuclei? From the studies of Klieneberger-Nobel (1945) and Delaporte (1950) it is evident that the chromatinic masses in *Bacillus* and *Clostridium* may be quite numerous, and other masses may be below the limit of resolution of the light microscope. Therefore, the number of a few to twenty cell sap vacuoles reported by Knaysi *et al.* (1950) may not

be unreasonable for nuclear material. If indeed these cells are multicellular as Bisset (1953) stated, no statement can be made about the number of vacuoles/cell until the number of cross-walls is determined. The cross-walls do not show in electron micrographs of *Mycobacterium* without special treatment (Bisset, 1953). If these vacuoles should be nuclear, then the small dense organelles found by Knaysi *et al.* (1950) in mycobacteria would be intranuclear and would have the same relationship to these cells as the organelles of myxobacteria.

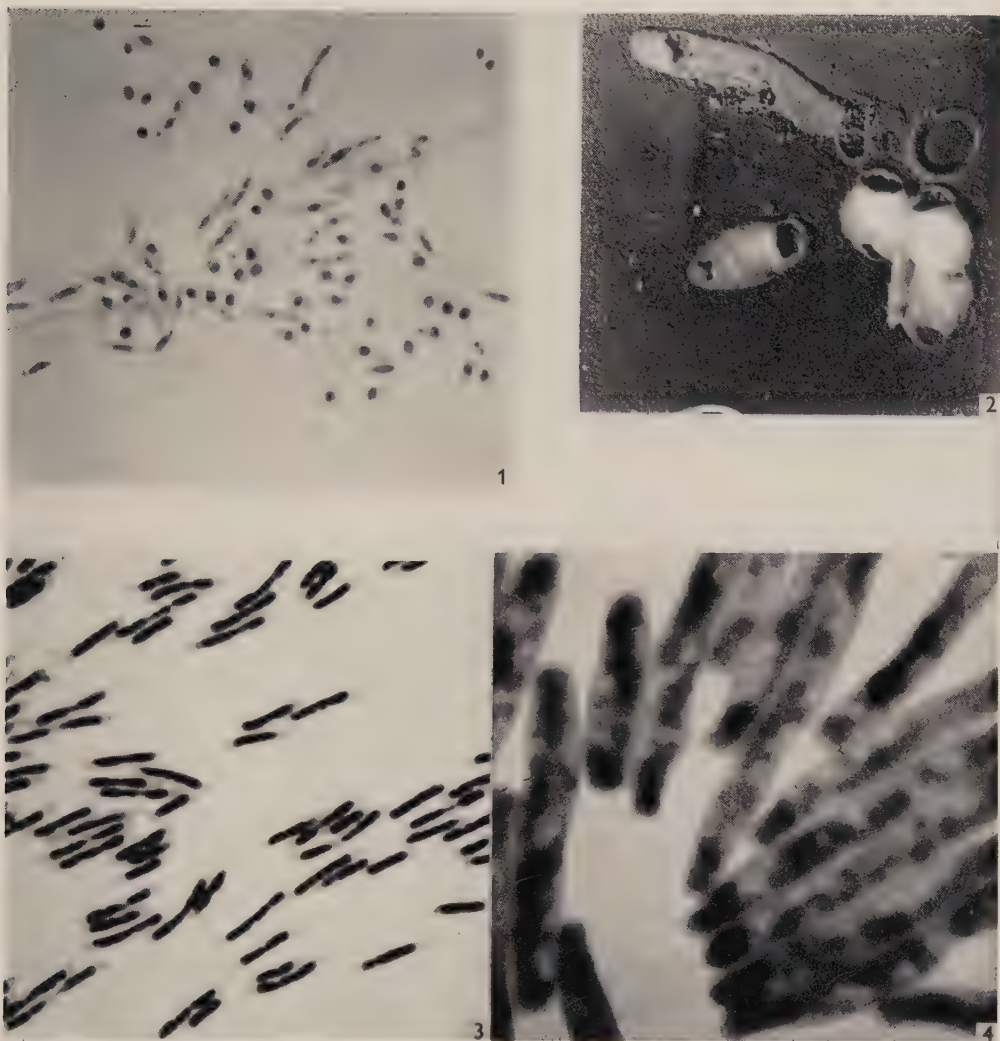
Germinating microcysts of *Myxococcus fulvus* show the organelles when no nuclear structures are visible (cf. Pl. 1, fig. 2). If at other stages of development these organelles are associated with the nucleus, then it is possible at this stage also. The thickness of organisms, either eubacterial or myxobacterial, may prevent the detection of a relatively transparent nucleus, but the dense organelles may still be seen. The electron-dense structures in mycobacteria and other bacteria which Mudd (1953) considered to be mitochondria had the following dimensions: *Mycobacterium* spp. $2.0 \times 0.5 \mu$. with spherules $0.02-0.05 \mu$.; *Corynebacterium* spp. $0.7-0.1 \mu$. or less; *Escherichia coli* lysed by phage $0.4-0.2 \mu$.; *Micrococcus cryophilus* $0.2-0.1 \mu$. The organelles occurring in *Myxococcus fulvus* are of the range of $0.05-0.10 \mu$. Their presence in the nucleus or on the nuclear membrane indicates that these structures in *M. fulvus* are not mitochondria.

The observations reported here lend support to the hypothesis that the nucleus in *Myxococcus fulvus* is surrounded at all times by a nuclear membrane. In young rapidly growing cells, karyokinesis is far in advance of cytokinesis; therefore the nuclear apparatus in these cells is very irregular and shows much lobation and many strictures. Hence little importance can be attached to the number of chromatinic bodies present in a given cell. The above hypothesis does not require a separate interpretation for eubacteria, since it is in keeping with the observations of Chapman & Hillier (1953). If typical mitosis were to occur during multiplication, it would be expected that resting cells with an interphase nucleus might be observed. Resting cells of *M. fulvus* were not seen, however, except in the intermediate or fruiting body region. Cells in these areas are probably in the phase of negative growth acceleration or the stationary phase. There are several possibilities to account for the bilobed nucleus. It may be a binucleate cell which is dividing, and the division might be delayed, leading directly to the formation of two microcysts. Another possibility is that each lobe of the bilobed vegetative nucleus contains the n number of chromosomes and that before going into the resting state the nucleus must acquire the $2n$ number; this would, of course, be autogamy. Bilobed nuclei have also been reported in eubacteria. They were demonstrated in *Escherichia coli* and other organisms by Robinow (1947), in preparations of *E. coli* treated with ribonuclease by Tulasne & Vendrely (1947), and were considered to be of sexual significance by Bisset (1950).

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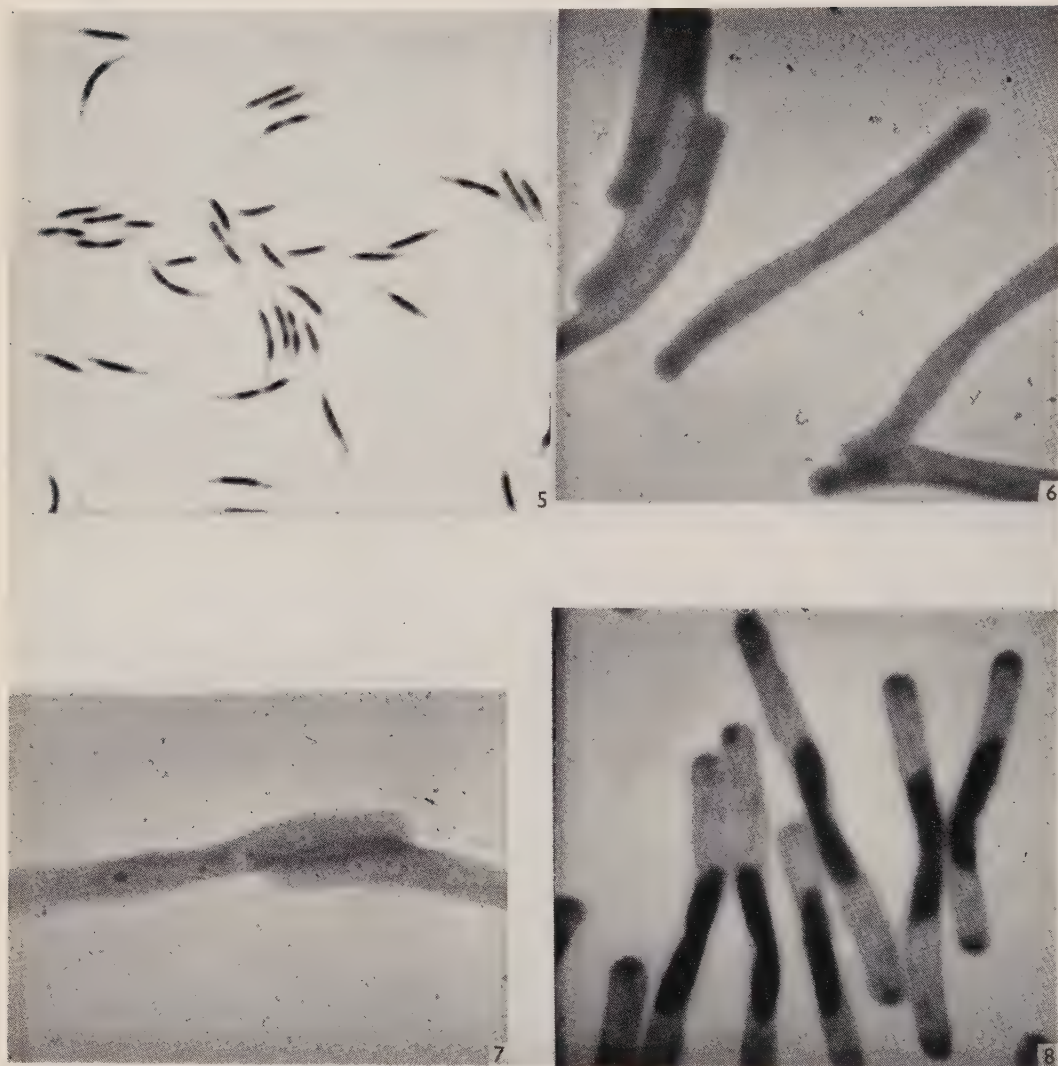
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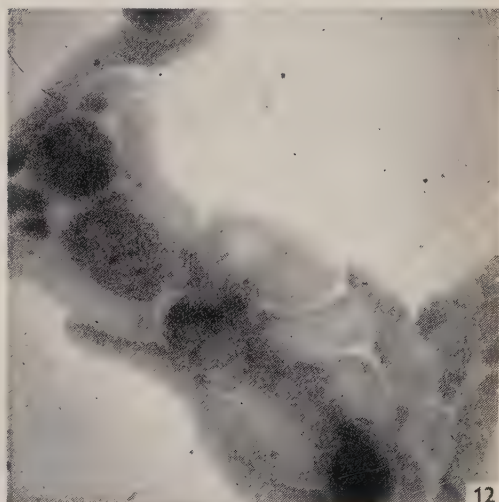
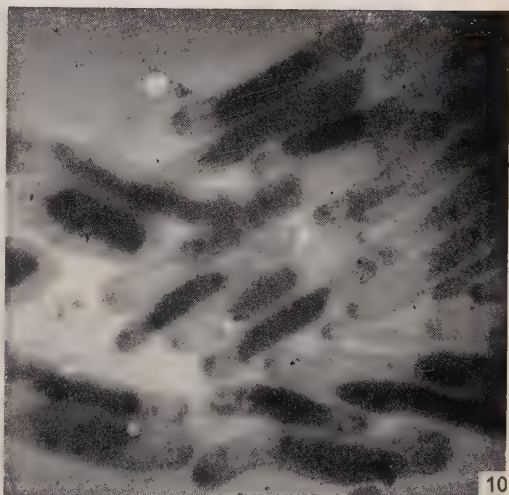
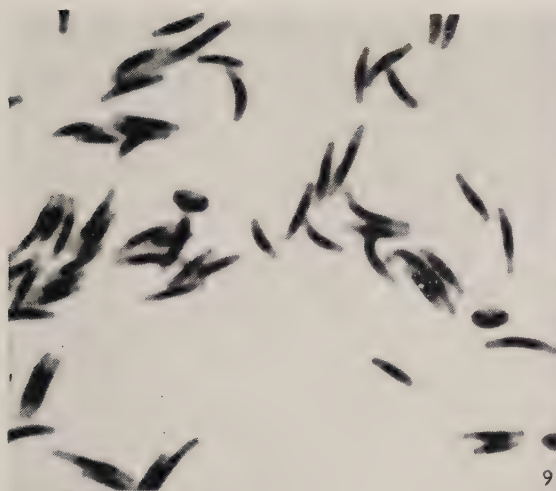


M. E. LOEBECK AND E. J. ORDAL—THE NUCLEAR CYCLE OF *MYXOCOCCUS FULVUS*.
PLATE 1

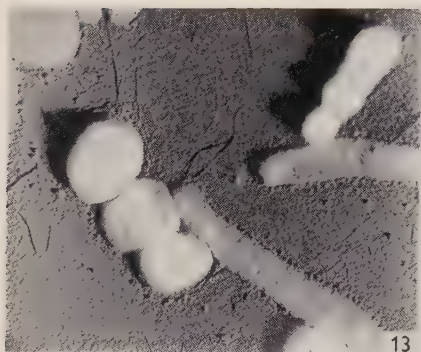
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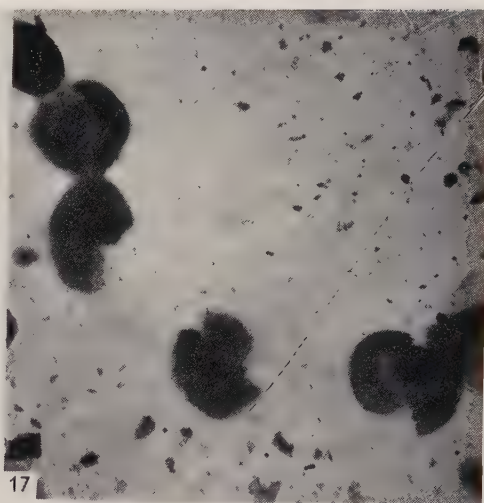
M. E. LOEBECK AND E. J. ORDAI.—THE NUCLEAR CYCLE OF *MYXOCOCCUS FULVUS*.
PLATE 2



M. E. LOEBECK AND E. J. ORDAL —THE NUCLEAR CYCLE OF *MYXOCOCCUS FULVUS*.
PLATE 3



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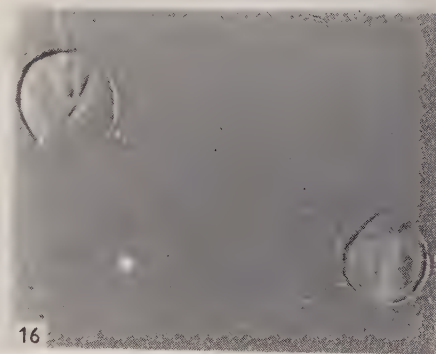
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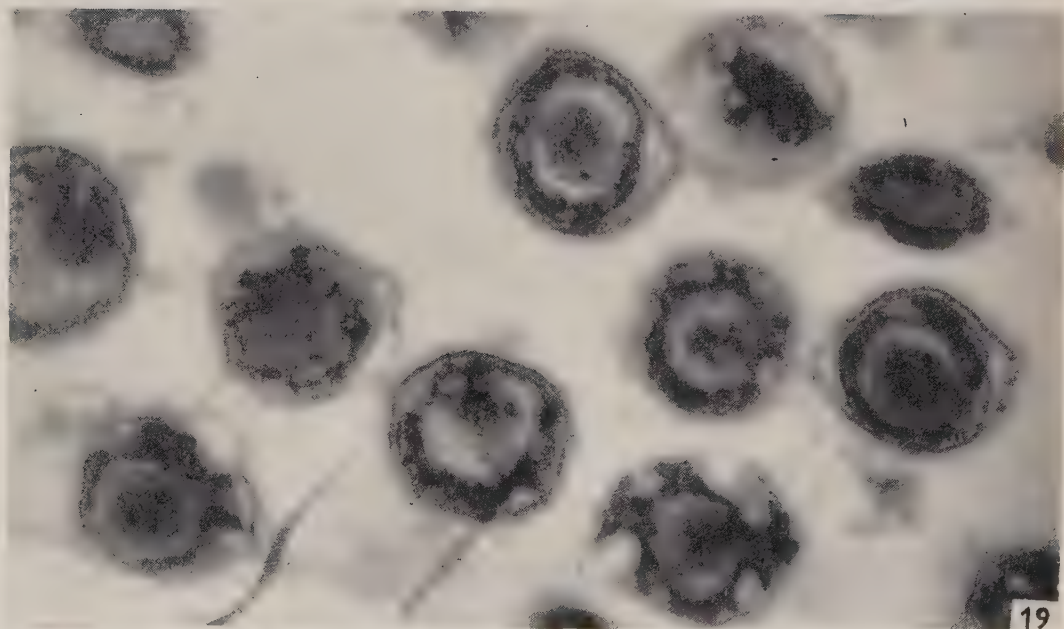
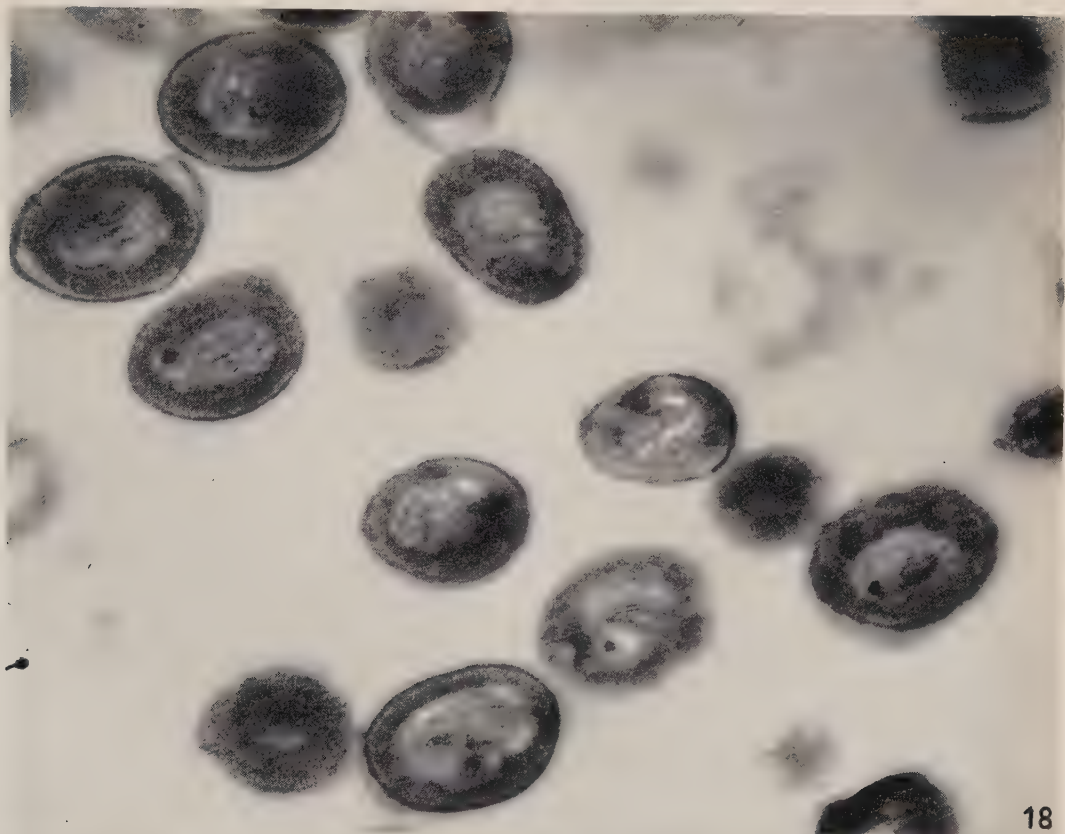
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M. E. LOEBECK AND E. J. ORDAL—THE NUCLEAR CYCLE OF *MYXOCOCCUS FULVUS*.
PLATE 5

EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Germinating microcysts, treated with HCl, followed by crystal violet. $\times 3000$.
Fig. 2. Germinating microcysts. Grown on dung agar for 5 hr. Picked up with collodion film. Ni shadowed. $\times 11,500$.
Fig. 3. Advancing swarm cells stained with Robinow-Giemsa method. $\times 3000$.
Fig. 4. Advancing swarm cells grown on collodion film over dung agar. Fixed with OsO_4 . $\times 11,500$.

PLATE 2

- Fig. 5. Intermediate cells by Robinow-Giemsa stain. $\times 3000$.
Fig. 6. Intermediate cells from 5-day culture. Formvar film over dung agar. OsO_4 fixed. $\times 11,500$.
Fig. 7. Intermediate cells from 5-day culture. Formvar film over dung agar. Not fixed. $\times 11,500$.
Fig. 8. Intermediate cells from culture grown $2\frac{1}{2}$ days on formvar film over dung agar. Not fixed. $\times 11,500$.

PLATE 3

- Fig. 9. Mature cells stained with Robinow-Giemsa stain. $\times 3000$.
Fig. 10. $3\frac{1}{2}$ -day culture grown on formvar film over dung agar. $\times 11,500$.
Fig. 11. Culture grown on formvar film over dung agar. Not fixed. $\times 11,500$.
Fig. 12. 5-day culture grown on formvar film over dung agar. Not fixed. $\times 11,500$.

PLATE 4

- Figs. 13, 14. Cells grown on dung agar, fixed with OsO_4 *in situ*, picked up with formvar film and chromium shadowed.
Fig. 15. Shattered microcysts, fixed with OsO_4 . $\times 11,500$.
Fig. 16. Outer shell of microcyst. Ni shadowed. $\times 11,500$.
Fig. 17. Shattered inner shell. $\times 11,500$.

PLATE 5

- Figs. 18, 19. Ultra-thin sections of microcysts. $\times 28,000$.

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The Influence of Oxygen and Arginine on the Motility of a Strain of *Pseudomonas* sp.

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SUMMARY: The motility of a strain of *Pseudomonas* sp. was activated by molecular oxygen or by arginine. In the presence of sufficient oxygen to support motility, arginine was not required for this purpose. In the absence of sufficient oxygen, arginine supported motility and was broken down to ornithine; there is not yet enough evidence to indicate whether this breakdown supplied the energy for such arginine-activated motility. In the presence or absence of arginine, the organism exhibited chemotaxis towards an optimum oxygen concentration which was less than that at the air/suspension interface.

As part of another investigation some heat-killed tubercle bacilli were disintegrated with small glass beads and the resulting material stored in the refrigerator at 4°. Some weeks later it was observed that the turbidity of the suspension had markedly increased, and a drop was examined microscopically on a glass slide under a coverslip. This revealed contaminating bacilli which at first were highly motile throughout the preparation, but after a few minutes lost their motility abruptly except around bubbles of air trapped under the coverglass. The organism was isolated and experiments were undertaken to determine the factors responsible for its selective motility; some of the results of these investigations have already been briefly reported (Preston & Sherris, 1955). The present paper is concerned with a more detailed description of those experiments and the results of further work.

METHODS

Organism used. The organism studied had the following characters: short Gram-negative rod, usually with a single polar flagellum, but sometimes lophotrichate or amphitrichate; viscid growth on nutrient agar at 23°, 48 hr. colonies 1-2 mm. diameter; turbidity in broth most marked near the surface, viscid deposit in older cultures; green fluorescent water-soluble pigment produced; gelatin liquefied; urease produced; nitrate not reduced to nitrite; indole and hydrogen sulphide not produced; alkaline reaction in litmus milk; growth in Koser's citrate; acid produced from glucose and sucrose, but no fermentation of lactose, maltose, salicin, mannitol, dulcitol; strict aerobe; good growth at 23°-30°, no growth at 37°.

The possession of these characters presents no difficulty in assigning this strain to the genus *Pseudomonas*. Descriptions of species within the genus, however, are far from complete; excluding the plant pathogens (since we have no information on its pathogenicity) this organism most closely resembles *P. viscosa* as described in *Bergey's Manual* (1948), and the above properties

differ from this description only in the inability of our strain to grow anaerobically. The organism used here will subsequently be referred to as 'the pseudomonad'.

Preparation of bacterial suspension. After overnight growth of the pseudomonad on nutrient agar at 23°, the bacteria were carefully removed from the surface of the medium and suspended in distilled water; the viscosity of these young cultures was not sufficient to prevent the ready preparation of homogeneous suspensions. The concentration of organisms in such suspensions was standardized so that the final preparation when mixed with other reagents would be equivalent in turbidity to Brown's opacity tube no. 5 ($c. 4 \times 10^9$ organisms/ml.). Unless otherwise stated, all preparations were adjusted to pH 7.2 with Kolthoff's buffer (final concentrations: 14.5 mM-potassium dihydrogen phosphate, 5.25 mM-borax).

Preparations for microscopical examination. Flat capillary tubes at least 50 mm. long were prepared from glass tubing by a modification of the method of Wright & Colebrook (1921). The material to be examined for motility was drawn into these tubes, leaving at one end a space containing air or some other gas. The tubes were then sealed, mounted on microscope slides with plasticine and examined under the 4 mm. objective. Resolution was excellent and distances from the meniscus were readily measured with the vernier on the microscope stage.

Gases other than air were introduced into the preparations by the following method. One end of the capillary tube was attached by rubber tubing to a cylinder of the appropriate gas and the other end held beneath the surface of a bacterial suspension. After allowing sufficient time for the air in the tube to be entirely replaced by the gas, the cylinder was closed and the rubber tubing squeezed and then released to withdraw a column of suspension into the capillary tube. The tube was then sealed at both ends, leaving the bacterial suspension in contact with the gas at the end which had been attached to the cylinder.

Estimation of arginine, ornithine and ammonia. Arginine concentrations were measured colorimetrically by the method of Sakaguchi (1950). Ornithine concentrations were determined by the method of Chinard (1952) in which ornithine reacts with ninhydrin in an aqueous acetic + phosphoric acid solution at 100° to give a red colour. Ammonia estimations were made by the phenol hypochlorite method of Lubochinsky & Zalta (1954). All colorimetric readings were made with a Hilger 'Biochem' absorptiometer.

RESULTS

Activation of motility by oxygen

The influence of air on motility. When aqueous suspensions of the pseudomonad were introduced into flat capillary tubes the organisms were at first actively motile throughout the length of the liquid; but after several minutes motility ceased abruptly except in a zone extending for about 3–4 mm. from the air space. By taking great care not to aerate the suspension during

transfer to the capillary tube, the duration of general motility could be decreased to 2–3 min., whereas previous deliberate and prolonged aeration enabled it to continue up to 80 min. In the zone near the air bubble, however, the organisms remained motile for many days at room temperature, the actual duration depending on the volume of the air space. These findings indicated that motility was dependent upon some expendable component of the air. This was confirmed by the observation that when a capillary tube was reopened after all motility had ceased, the organisms immediately became actively motile again in the zone near the air space. One such tube was opened and resealed five times over a period of 2 months, and motility was re-established on each occasion.

The effect of other gases on motility. When oxygen was introduced into a capillary tube in place of air, general motility throughout the length of the suspension again ceased after a few minutes, but the residual motile zone now extended for about 8 mm. from the meniscus. The introduction of nitrogen, hydrogen or carbon dioxide into the capillaries, however, failed to support motility at any point in the suspension after the initial brief period of general motility had ceased. These findings indicated that the initial general motility was due to dissolved oxygen, and that the continuing motility in the zone near the air/suspension interface was due to the oxygen content of the air. These phenomena will be referred to respectively as 'general aerobic motility' and 'superficial aerobic motility'.

Chemotactic effect of oxygen. In the initial observations on suspensions which were examined on glass slides under coverslips, it was noticed that the bacteria tended not only to exhibit maximum motility around air bubbles but also to accumulate there. It was not clear whether this apparent movement of organisms towards the air was due to some physical effect such as surface tension or even a lateral expansion of the bubble, or whether it constituted a true chemotaxis. The following results, obtained with suspensions in flat capillary tubes, indicate that oxygen does exert a chemotactic effect on the organisms. Approximately 20 min. after setting up a preparation, the zone of superficial aerobic motility was seen to be differentiated into three parts. Nearest the air space was a section in which the organisms were moderately motile, and adjacent to this was a zone of increased motility and apparent concentration of the organisms. Beyond this was a small zone of definite rarefaction separating the motile from the non-motile portions of the suspension; this zone of rarefaction was readily visible macroscopically as well as microscopically.

After several days the non-motile organisms settled to the bottom of the tube and the highly motile and concentrated zone became increasingly apparent as a sharply defined band. In one experiment such a preparation was observed daily for 3 weeks. After 6 days the band was 0.1 mm. across and was found to move gradually towards the meniscus (see Table 1*a*). Just before it reached the meniscus the tube was reopened and within a few minutes the band was moving back along the tube away from the opened air space (see Table 1*b*). This rapid change in position of the band of highly motile organisms

was not due to resuspension of deposited bacteria in a region of optimal oxygen concentration, for had this been the case, large numbers of resuspended organisms would have been left behind as the band retreated from the meniscus. This did not occur, for the zone between the meniscus and the moving band was almost free from suspended bacteria. The highly motile organisms which formed the band were thus moving *en masse* as oxygen diffused into the suspension, and were exhibiting chemotaxis towards a concentration of oxygen less than that in air and apparently optimal for their activity. This chemotactic phenomenon in the zone of superficial aerobic motility was also noted when arginine was present in the bacterial suspension.

Table 1. *Chemotactic effect of oxygen*

(a) Movement of narrow band of highly motile pseudomonads towards air/suspension interface in sealed capillary tube

Time from sealing tube (days) ...	6	11	17	23
Distance of band from meniscus (mm.)	2.9	1.7	1.0	0.2

(b) Movement of band away from meniscus after reopening tube

Time from reopening tube (min.) ...	0	4	14	42	107
Distance of band from meniscus (mm.)	0.2	0.6	1.2	1.9	2.0

Activation of motility by arginine

Maintenance of motility by nutrient broth. The observations so far recorded were made on aqueous suspensions of the pseudomonad; but different results were obtained when a double-strength suspension was mixed with an equal volume of nutrient broth before examination in a capillary tube. Observations were now confined to those parts of the suspension which were more than 20 mm. from the air space, i.e. regions where motility in aqueous suspensions ceased after a few minutes. The organisms in nutrient broth became non-motile, following the period of general aerobic motility, at approximately the same time as those in aqueous suspensions; but, after a pause of a few seconds, the broth suspension again exhibited motility which continued for at least 40 min. (see Fig. 1). These findings led to an examination of the constituents of broth in a search for some factor capable of reactivating this 'deep' motility.

Investigation of factors in broth responsible for reactivating motility. It seemed possible that the restored deep motility seen in broth suspensions might be due to the gradual release of oxygen or to a higher oxidation-reduction potential than that in aqueous suspensions. However, the results obtained with untreated broth could be reproduced with boiled broth, Brewer's medium, or broth containing 0.1% (v/v) mercaptoacetic (thioglycolic) acid. These findings, together with the brief pause in motility which occurred in broth, indicated that the restored motility was due to mechanism different from that which was activated by oxygen. Further search showed that digest broth or a 1% (w/v) solution of peptone was active but meat extract (without added peptone) and the supernatant fluid from an overnight broth culture of the

organism were without effect. This suggested that the responsible factor was a product of protein breakdown used by the organism during growth, and thus led to an investigation of amino acids.

Twenty-two amino acids were tested separately in final concentrations of 5, 0.5, and 0.05 mM. They were: glycine, DL-alanine, DL-valine, DL-leucine, DL-isoleucine, DL-serine, DL-threonine, L-cysteine, DL-methionine, L-aspartic acid, L-asparagine, L-glutamic acid, DL-lysine, L-arginine, L-citrulline, L-ornithine, glycoeyamine, creatine, DL-histidine, L-proline, L-hydroxyproline, DL-

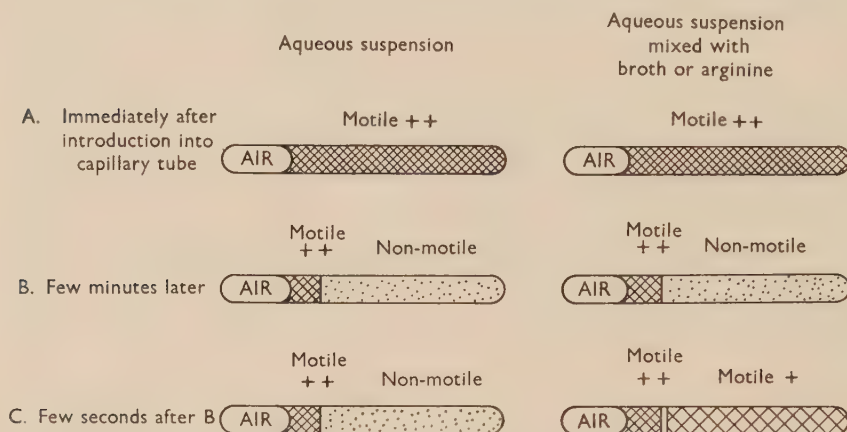


Fig. 1. The effect of air, and broth or arginine, on the motility of suspensions of the pseudomonad when studied in capillary tubes.

phenylalanine. L-Tyrosine and DL-tryptophan were tested in concentrations of 0.5 and 0.05 mM. Of these amino acids only L-arginine was capable of restoring deep motility, and at 0.5 mM maintained it for 45 min. This restored motility activated by arginine and occurring after the brief pause which followed the period of general aerobic motility will be referred to as 'arginine-activated motility'.

Quantitative effect of arginine. The duration of arginine-activated motility was directly proportional to the concentration of arginine in the suspension

Table 2. *Relationship between duration of arginine-activated motility and concentration of arginine*

Final concentration of arginine \times 3 mm ...	2 ⁰	2 ⁻¹	2 ⁻²	2 ⁻³	2 ⁻⁴	2 ⁻⁵	2 ⁻⁶
Duration of motility (min.)	182	86	43	22	12	7	2

over the range 3 mM to 50 μ M (see Table 2). A linear relationship did not apply, however, when much higher concentrations of arginine were used, for a fourfold increase in arginine concentration (from 3 to 12 mM) resulted in an increase in the duration of motility from 3 hr. to more than 21 hr. This proportionately greater increase in the duration of motility was associated with more sluggish movement of the organisms and with the development of a markedly alkaline reaction (pH > 8.2) in the capillaries. An

experiment was therefore made to test the effect of alterations in pH value on the duration of arginine-activated motility. The results (see Table 3) showed that a reaction more alkaline than pH 7.6 caused an increase in the duration of motility, an increase which was again accompanied by more sluggish movement of the bacteria.

Table 3. *The influence of pH value on the duration of arginine-activated motility*

Arginine concentration 0.4 mM.						
pH of buffered suspension ...	6.6	7.0	7.4	7.8	8.2	8.6
Duration of motility (min.)	21	20	22	23	42	> 120

The relationship of arginine breakdown to motility. A standard suspension of the pseudomonad was made in buffered saline consisting of M/30 phosphate buffer (pH 7.2) and 0.5% (w/v) NaCl. After allowing equal volumes of this suspension and of a solution of arginine (1 mM) in buffer to equilibrate in a water bath at 23°, they were mixed and quickly distributed in 4 ml. amounts to narrow tubes (9 × 90 mm.). The tubes were incubated at 23°, removed at suitable intervals, and the contents allowed to drain into 1 ml. of 5% (w/v) trichloroacetic acid. The organisms were removed by centrifugation and the arginine concentrations in the supernatant fluids determined. The motility of the suspension was examined by filling capillaries from the tubes at intervals and examining them microscopically. The conditions under which the reaction was allowed to proceed were chosen to resemble those found in capillary tubes on the microscope stage, i.e. a small surface area in contact with air and a temperature of 23°. That this end was achieved is shown by the fact that motility ceased at the same instant in all capillaries, though these were filled from the reaction mixtures at various time intervals. This did not occur when the mixtures were incubated in wide tubes, or at a different temperature from that found on the microscope stage.

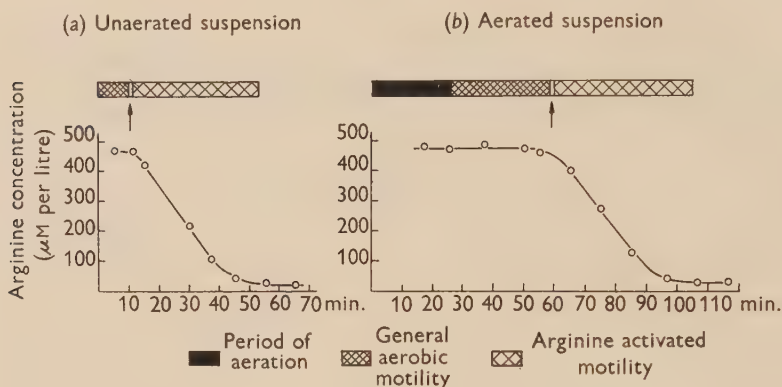
The results show (Fig. 2a) that little or no breakdown of arginine occurred during the period of general aerobic motility. After the brief cessation of motility in the deeper parts of the capillary tubes arginine-activated motility began and arginine was decomposed at a steady rate. Motility ceased when all the arginine had been broken down.

The relationship between arginine breakdown and arginine-activated motility was found to be independent of the duration of the preceding period or general aerobic motility. When suspensions containing arginine were briskly aerated before distribution to narrow tubes, there was no breakdown of arginine during aeration or the subsequent period of prolonged general aerobic motility. The period of arginine-activated motility still corresponded, however, to the period of arginine breakdown (Fig. 2b).

The fate of arginine

Chromatographic analysis. A bacterial suspension prepared in M/80 phosphate buffer (pH 7.2) was incubated in the presence of 20 mM arginine at 18°. Samples were removed at hourly intervals for 6 hr., centrifuged, and the

supernatant fluids examined by chromatography. The chromatograms were run on Whatman no. 1 paper with an ascending boundary, using phenol saturated with water as the mobile phase. On spraying the chromatogram with a ninhydrin solution spots corresponding to arginine were found in all samples, while others, corresponding to ornithine, appeared in samples derived from reaction mixtures which had been incubated for more than 1 hr. No spots developed corresponding to citrulline or any amino acid other than arginine and ornithine. Similar chromatographic methods were applied to mixtures of bacterial suspension and citrulline. There was no evidence of citrulline breakdown, and no ornithine was produced.



At the point indicated by the arrow motility ceased completely for a few seconds

Fig. 2. The relationship of arginine breakdown to motility.

Quantitative colorimetric determinations. Arginine (final concentration $525 \mu\text{M}$) was added to a twice-washed suspension of the pseudomonad in buffered saline. The reaction mixtures were maintained at 23° ; 4 ml. samples were removed at intervals and allowed to drain into 1 ml. 5% (w/v) trichloroacetic acid and the mixtures heated at 100° for 15 min. to ensure that the products of the reaction were extracted from the organisms. These were then removed by centrifugation and colorimetric estimations of arginine, ornithine and ammonia were made on the supernatant fluids. A suspension without added arginine was similarly treated to obtain blank values.

The results in Table 4 show that, within experimental error, all the arginine broken down could be accounted for as ornithine and ammonia. The possibility was considered that the initial breakdown of arginine was to ornithine and urea, and that the urease elaborated by the organism decomposed urea as it was formed. Urease activity in culture was, however, detected only after incubation on a urea-containing medium for 3 days, whereas there was no detectable breakdown of added urea (concentration $500 \mu\text{M}$) within 1 hr. by suspensions of the organism. Moreover, treatment of the supernatant fluids with urease failed to increase the amount of ammonia present. These findings

indicate that the hydrolysis of the arginine was brought about by an enzymic process similar to the arginine dihydrolase system of Hills (1940), rather than by the mammalian type of arginase.

Table 4. *Relationship of ornithine and ammonia production to arginine breakdown*

Time from addition of arginine (min.)	Arginine decomposed (μ mole/l.)	Ammonia formed (μ mole/l.)	Ornithine formed (μ mole/l.)
0	0	2×0	0
20	25	2×45	25
40	245	2×250	230
60	495	2×480	495
80	525	2×520	520

Blank values. No arginine or ornithine detected. Ammonia 25μ mole/l.; the figures in the table were obtained by subtraction of the blank reading.

The effect of other reagents on motility

Other reagents were added to equal volumes of bacterial suspension to test for their ability to prolong general aerobic motility or to reproduce the restored deep motility given by arginine. All materials added gave negative results. They included: adenosine triphosphate (2.5 mM to $0.25 \mu\text{M}$); urea (25 mM to 0.25 mM); ornithine (1 mM) + ammonium carbonate (1 mM) to simulate the end products of arginine breakdown by arginine dihydrolase (Hills, 1940); potassium nitrate (1% to 0.001% , w/v); methylene blue (0.01% to 0.0001% , w/v); glucose (1% to 0.01% , w/v), and modified Proskauer and Beck's medium (quoted by Jensen, 1954) which is a defined medium containing but one amino acid, asparagine, and which supported excellent growth of the pseudomonad. A mixture of adenosine triphosphate (0.75 mM to $7.5 \mu\text{M}$) + arginine (0.4 mM) had no more effect on the duration of arginine-activated motility than that produced by arginine alone.

DISCUSSION

The findings recorded here show that, in aqueous suspensions of the pseudomonad studied, motility may be activated under aerobic conditions by oxygen, or under relatively anaerobic conditions by arginine. These two processes are qualitatively different, for very little, if any, arginine is broken down during the period of aerobic motility, and motility activated by arginine does not begin until a second or two after the oxygen content of the suspension has fallen below the minimum level needed for aerobic motility. The requirements for aerobic motility reflect those for growth and metabolism of the organism, but arginine is not needed as a nutrient nor does its addition to solid or fluid media allow growth to occur under anaerobic conditions.

As definite information is not at present available about the biochemical processes which supply energy for flagellar activity, it is worth considering how arginine might contribute to this process. One possibility is that the

energy may be liberated directly by the breakdown of arginine to ornithine; another, more in keeping with present knowledge of other systems which involve biological movement, is that energy-rich phosphates are produced during the arginine breakdown and that they furnish the immediate source of energy. A clue as to how this might be brought about is given by the work of Slade, Doughty & Slamp (1954) who showed that cell-free extracts of a strain of *Pseudomonas* sp. isolated from soil converted citrulline to ornithine, carbon dioxide and ammonia in the presence of bivalent ions, inorganic phosphate and adenosine diphosphate (ADP) or adenylic acid. Breakdown of citrulline under these conditions was associated with the formation of adenosine triphosphate (ATP) due to the esterification of the phosphate supplied.

The pseudomonad we have studied resembles that of Slade and his colleagues in that it breaks down arginine, but not ornithine, and it may be that citrulline is produced as an intermediate product as in the case of the cell-free extracts of *Streptococcus faecalis* studied by Slade (1953). The fact that we have been unable to detect citrulline chromatographically, that it had no effect on motility and that it was not broken down by suspensions of whole organisms, cannot be held to exclude the possibility that ATP, produced by the citrulline-ureidase reaction of Slade and his colleagues, furnishes the immediate source of energy for motility of our strain. Slade & Slamp (1952) showed that, in the case of *S. faecalis*, whole organisms were unable to break down citrulline whereas cell-free extracts were active (Slade, 1953). It is possible that the lack of activity of citrulline in our experiments was due to its failure to pass into the pseudomonad.

The possibility may also be considered that arginine is required for some other system and that its breakdown, while limiting the duration of its activity, plays no part in supplying energy for motility. X-ray diffraction studies and chemical analysis of isolated bacterial flagella (Astbury & Weibull, 1949; Weibull, 1950, 1951) showed that they consist of a protein 'flagellin' (Astbury, Beighton & Weibull, 1955) which belongs to the keratin-myosin-epidermin-fibrinogen group of fibrous-elastic proteins, and these workers conclude that bacterial flagella are effectively monomolecular muscles. Arginine phosphate in invertebrate muscle plays the same role as creatine phosphate in vertebrate muscle. In contraction of invertebrate muscle the reaction $\text{arginine phosphate} + \text{ADP} \rightarrow \text{arginine} + \text{ATP}$ proceeds from left to right as ATP is broken down by adenosine triphosphatase to liberate energy. When contraction ceases arginine is re-phosphorylated by energy-rich phosphates derived from anaerobic glycolytic processes. If a similar system obtains for storing and providing energy for flagellar activity it is possible that, under conditions in which an arginase is active, the addition of arginine would temporarily allow re-synthesis of sufficient arginine phosphate for motility to proceed.

Both the mechanisms of action of arginine considered above involve the assumption that ATP is the immediate source of energy for flagellar activity; analogy with other biological systems which involve movement (e.g. muscle contraction; mitosis; motility of trypanosomes and spermatozoa; see Weber,

1955) suggests that this is probably the case. Some evidence on this has been given by De Robertis & Franchi (1951) who reported contraction of the flagella of *Bacillus brevis* in the presence of ATP, and by De Robertis & Peluffo (1951) who found that ATP enhanced the motility of *Proteus vulgaris*. The lack of activity of ATP in our experiments may, as suggested for citrulline, be due to its failure to pass the permeability barrier of the pseudomonad.

The chemotactic effect of oxygen appears to merit further study, for similar observations were made by Engelmann (1882, 1894) with a variety of bacteria. In his later experiments algae were incorporated in the bacterial preparations, and by varying the amount of light impinging on them he was able to vary the local production of oxygen during photosynthesis, and thus obtained a most convincing demonstration of chemotaxis. Some of his bacteria aggregated in the immediate vicinity of the algae, but others were concentrated in a band some distance from the source of oxygen, producing a very similar phenomenon to that observed in our experiments.

Our thanks are due to Dr C. Weibull for drawing our attention to the work of Engelmann.

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The Kinetics of the Mating Process in *Escherichia coli*

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SUMMARY: When broth cultures of donor (HfrH) and recipient (F⁻) strains of *Escherichia coli* K-12 are mixed, zygotes are formed by the transfer of part of the donor chromosome to the recipient cell. The donor parent thus becomes dispensable as soon as transfer is accomplished. The kinetics of zygote formation can therefore be studied by treating samples, removed at intervals from a parental mixture, with virulent bacteriophage to which only the donor parent is susceptible. Only zygotes already formed at the time of treatment can segregate a recombinant cell. A lag of 8-10 min. precedes a linear rise in the number of zygotes when selection is made for inheritance of the donor nutritional markers T⁺ L⁺ only. The formation of zygotes inheriting the marker Lac⁺ as well as T⁺ L⁺ shows a lag of about 18 min. These lag periods represent the times required for the genes T⁺ L⁺ and Lac⁺, respectively, to enter the F⁻ cells and confirm the finding of Wollman & Jacob (1955) that chromosome transfer is an oriented process and that the donor genes penetrate the F⁻ cell in the same order as their arrangement on the chromosome. The process of zygote formation in the equivalent F⁺ × F⁻ cross has also been studied by the phage method. Although the yield of T⁺ L⁺ recombinants is *c.* 2 × 10⁴ times less than in the Hfr × F⁻ cross under the same conditions, the times of entry of the donor genes T⁺ L⁺ and Lac⁺ are the same in both crosses. In the Hfr × F⁻ cross, significant zygote formation does not occur in unsupplemented buffer but requires the presence of both glucose and sodium aspartate. Zygote formation is a temperature-dependent process which occurs in the absence of multiplication of either parent and is unaffected by the presence of deoxyribonuclease. The number of zygotes (and therefore of recombinants) formed in a given time is a function of two independently variable factors: (i) the frequency and intimacy of chance contacts; (ii) the speed of chromosome transfer which is related to energy production. Decrease of temperature from 37° to 32° about doubles the time required for any given Hfr gene to be transferred to an F⁻ cell. Alteration of the parental population density, or the pH values of the medium (Fisher, 1957*b*), does not affect the times of entry of Hfr genes into the F⁻ cells but does modify the rate of effective contact formation. Segregation of haploid recombinant cells from Hfr × F⁻ zygotes, at 37°, takes place in nutrient broth at about 140 min., and on minimal agar at about 160 min., after mixing the parental suspensions. The phenotypic expression of resistance to sodium azide, inherited from the Hfr parent, commences shortly after the zygotes are formed and becomes complete just before segregation; resistance to phage T₁, however, is not expressed at all until the time of segregation, and requires four generations of the recombinant segregants for completion.

Previous studies of the kinetics of recombination in *Escherichia coli* K-12 were undertaken by Nelson (1951) and by Marguerite Vogt (1952, personal communication) with the aim of obtaining quantitative information about the nature of the cellular contacts which initiated recombination, and of defining

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the optimal conditions for mating. Both these studies preceded the discovery that recombination is mediated by contact between cells of different mating type. The cells of one type (F^+) act as genetic donors and of the other type (F^-) solely as recipients, so that mating involves a one-way transfer of genetic material from F^+ to F^- cells. Crosses between F^- cells are sterile (Hayes, 1952; Lederberg, Cavalli & Lederberg, 1952; Cavalli, Lederberg & Lederberg, 1953; Hayes, 1953 *a, b*).

The maximum yield of recombinants from $F^+ \times F^-$ crosses is of the order of one per 10^4 to 10^5 parental cells. However, mutant donor strains have been isolated which show a very much higher degree of fertility than the F^+ strain from which they were derived (Cavalli, 1950; Hayes, 1953 *b*). These strains are termed 'Hfr' on account of their high frequency of recombination. One of them (HfrH) regularly yields one recombinant prototroph per 10 F^- recipient cells when equal numbers of the two strains are mixed (Hayes; unpublished). Such a high frequency of recombination implies at least as high an efficiency of mating. In fact, conjugation between pairs of cells in mixtures of Hfr and F^- cultures is readily observable microscopically (Lederberg, 1956). $Hfr \times F^-$ crosses are thus well suited to kinetic analysis.

THEORETICAL BACKGROUND

The mating process begins when an effective contact is established between a donor and a recipient cell, and ends with the appearance of a haploid recombinant cell in which those characters inherited from the donor parent are functionally expressed. Several intervening steps can arbitrarily be distinguished. Once effective contact is made, the chromosomal contribution of the donor cells is transferred to the recipient cell to form a zygote. The function of the donor cell is then fulfilled and its further participation is not required. Within the zygote, a process formally analogous to crossing-over then occurs which leads to the creation of a recombinant chromosome.

Certain important assumptions may be made as to the constitution and behaviour of the zygote. First, genetic analysis of $F^+ \times F^-$ crosses reveals a continuous system of linkage uniting all of the large number of genetic markers of *Escherichia coli* K-12 (Clowes & Rowley, 1954; Cavalli-Sforza & Jinks, 1956) so that the haploid nucleus of this organism may be assumed to comprise a single chromosome. Secondly, the majority of the characters inherited by recombinants are derived from the recipient parent, suggesting that the donor cell usually contributes only part of its chromosome to the zygote (Hayes, 1953 *a, b*). This theory of partial transfer has recently been substantiated (see Wollman, Jacob & Hayes, 1956). The zygote therefore contains the complete chromosome of the recipient cell, but only part of the chromosome of the donor cell, and consequently cannot yield more than one viable recombinant cell, unless replication of the parental chromosomal contributions occurs before recombination. Thirdly, it may be concluded that the zygote does not usually divide before recombination since each recombinant colony, representing the selected progeny of a single zygote, tends to contain only cells of one genotype (Leder-

berg, 1947); if the zygote divided before recombination to produce two or more daughter zygote cells, each of these would often yield a different type of recombinant prototroph so that prototroph colonies stemming from single, fertilized recipient cells would tend not to be homogeneous.

Following recombination of genes within the zygote, segregation occurs so that a haploid cell possessing the recombinant chromosome is produced. Since this cell alone is able to multiply on the selective medium designed to demonstrate its occurrence, it will proceed to divide to form a colony of recombinant cells. But the cytoplasm and cell wall of the zygote are those of the F⁻ recipient cell so that not all of the genes inherited from the donor parent may be capable of functional expression immediately after segregation. The presence or absence of delay in the phenotypic expression of any character will depend upon the particular way in which the controlling gene determines the character. For example, a character which is directly expressed through synthesis of an enzyme may be expected to become manifest more rapidly than one, such as resistance to bacteriophage, which requires a radical reorganization of cell-wall structure.

In the work to be described, the stages of zygote formation, segregation and phenotypic expression in an Hfr \times F⁻ cross are experimentally defined and analysed.

DEVELOPMENT OF METHODS

The kinetics of zygote formation

In previous kinetic studies, such as that of Nelson (1951), suspensions of the two parental strains were mixed in a fluid medium and, at intervals thereafter, samples were withdrawn and plated on minimal agar which permitted the growth of prototrophic recombinants while suppressing that of the auxotrophic parents. When, in an experiment of this kind, the number of prototrophic colonies is plotted as a function of time, a curve is obtained which rises linearly from the origin. This curve must describe the rate at which irreversible unions are formed between donor and recipient cells in the mixed population, since the sole effect of diluting and plating the samples is to separate those cells which have not united firmly, and to prevent further contacts. The pairs of cells thus transferred to the minimal agar can continue to mate on the plate and may produce prototrophic recombinants. These unions must be established very rapidly since the curve expressing the rate of their formation begins to rise from zero time.

It is clear that this method can yield no information about the kinetics of zygote formation since it only scores the total number of mating pairs present in the population at any given time, which yield a prototrophic recombinant. It cannot distinguish between freshly formed unions and those which may already have mediated genetic transfer.

The basic function of the donor cell in recombination is to transfer part of its chromosome to the recipient cell to form the zygote. As soon as the zygote has been formed, but not before, the donor cell plays no further role in mating and is dispensable. If, therefore, it were possible to kill the donor cells rapidly

and selectively, in samples removed at intervals from a mating mixture, so that chromosomal transfer between mating pairs was prevented or arrested, recombinants arising from the treated mixture should be derived only from those zygotes which had already been formed at the time of treatment. One of the virulent bacteriophages of the 'T' series seemed to be an ideal agent for this purpose. These phages irreversibly inhibit the metabolic activities of the sensitive host cell almost immediately after they are adsorbed (Cohen, 1947), while bacterial strains resistant to them can easily be obtained by mutant selection.

One of the characteristic features of the Hfr donor strain used in this study is that only a limited number of Hfr genetic markers is inherited with significant frequency among prototrophic recombinants (Hayes, 1953*b*; Wollman & Jacob, 1954; see Fig. 1). Among the Hfr markers which are not inherited is sensitivity (or resistance) to phage T_3 which therefore seemed to be the ideal choice since, if the Hfr parent were sensitive and the F^- parent resistant, all the zygotes (i.e. fertilized F^- cells) and their prototrophic progeny would be resistant so that no distortion of segregation ratios would follow phage treatment. Preliminary experiments, however, revealed two disadvantages in the use of phage T_3 :

(1) Since the original auxotrophic Hfr donor strain was inherently resistant to phage T_3 , a sensitive Hfr derivative of this strain, obtained by recombination, had to be used instead. This strain had fewer differential genetic markers than the original strain so that genetic analysis of recombinants was limited.

(2) The high-titre phage T_3 preparations, necessary to ensure high multiplicity of infection, frequently contained sufficient host-range mutants to initiate visible lysis of the resistant F^- population under the experimental conditions then observed. For these reasons phage T_6 , to which the original Hfr strain (as well as the analogous F^+ strain, 58-161) is sensitive and in which host-range mutants have not been found (Doermann, 1953), was substituted for phage T_3 and was found satisfactory. Unlike phage T_3 sensitivity, the sensitivity to phage T_6 of the Hfr (or F^+) parent is normally inherited by *c.* 20 % of prototrophs (Fig. 1). This proportion was found to remain unchanged despite treatment of the parental mixture with phage T_6 since the subsequent dilution (or washing) of the treated mixture reduces to insignificance the probability of residual phage particles contacting sensitive recombinant segregants on the surface of the selective medium on which they are spread. The use of phage T_6 antiserum to neutralize surplus phage after treatment is, therefore, unnecessary.

Before studying the kinetics of zygote formation by means of the phage method, it is necessary to define the word 'zygote' in operational terms. Fig. 1 is a schematic representation of the chromosomes of the donor (Hfr or F^+) and recipient (F^-) strains employed, showing the relative positions of the genes controlling various characters to which reference will be made (see Cavalli-Sforza & Jinks, 1956). That part of the donor chromosome represented by an interrupted line is not transferred to the zygote by the Hfr strain. If the interrupted line is ignored, therefore, Fig. 1 portrays the constitution of an

average zygote from the Hfr \times F $^-$ cross. To demonstrate recombinants of any given class in such a cross, selection is made, in effect, for inheritance by the F $^-$ cell of the Hfr gene or genes which characterize this class (Hayes, 1953*b*). Thus on minimal agar supplemented with vitamin B₁ (thiamine) the F $^-$ cell cannot grow because it lacks the ability to synthesize threonine and leucine (T $^-$ L $^-$). When zygotes are plated on this medium, therefore, selection is, in effect, made for recombinants possessing an F $^-$ chromosome which has inherited that part of the donor chromosome carrying the closely linked genes T $^+$ and L $^+$, so that a prototrophic (T $^+$ L $^+$) recombinant cell is produced. When recombinants are selected in this way, on the basis of inheritance of T $^+$ L $^+$ alone, it is found that the frequency with which the other, unselected,

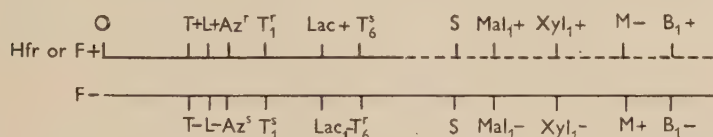


Fig. 1. The order of arrangement of various loci on the *Escherichia coli* K-12 chromosome (after Cavalli-Sforza & Jinks, 1956). The probable positions of the loci are only approximately represented since the map is only intended to clarify some concepts explained in the text. That part of the HfrH chromosome which is not transferred to the F $^-$ cell during mating is shown as an interrupted line. The symbols represent:

T = threonine	} synthesis	Az = sodium azide	} resistance or sensitivity	Lac ₁ = lactose	} fermentation
L = leucine		S = streptomycin		Mal ₁ = maltose	
M = methionine		T ₁ = phage T ₁		Xyl ₁ = xylose	
B ₁ = vitamin B ₁		T ₆ = phage T ₆			

'O' represents the extremity of the HfrH chromosome which first penetrates the F $^-$ cell (Wollman & Jacob, 1955). The phage T₃ locus is not marked since its position has not yet been mapped; but it is known to be situated on that part of the HfrH chromosome shown as an interrupted line.

markers are inherited among them varies. Thus about 90% inherit the Hfr marker Az^r (resistance to sodium azide), about 75% the marker T₁^r (resistance to phage T₁) and about 40% the marker Lac⁺ (ability to ferment lactose). Since the more closely two markers are linked together on the chromosome the greater is the likelihood that they will be inherited together, these frequencies are inversely related to the distance each marker is situated on the chromosome from the selective markers T⁺ L⁺, as Fig. 1 shows. Similarly, the F $^-$ cell does not ferment lactose (Lac⁻) so that if glucose is replaced by lactose in the minimal agar, selection is now made for another type of recombinant in which the F $^-$ chromosome acquires, by crossing-over, both T⁺ L⁺ and Lac⁺ from the donor parent. In such a case it is usually found that the whole donor chromosomal segment, TL—Lac, is inherited since the great majority of T⁺ L⁺ Lac⁺ prototrophic recombinants also possess the intervening unselected Hfr markers (Az^r and T₁^r).

In all early experiments defining the kinetics of zygote formation, selection was made for T⁺ L⁺ recombinants only. Thus, in these experiments, the term 'zygote' means, operationally, an F $^-$ cell to which the genes T⁺ L⁺

have been transferred and which is therefore potentially able to segregate a T+ L+ recombinant. It is unlikely, from the genetic data, that every zygote which receives the T+ L+ genes in fact produces a T+ L+ recombinant, so that the actual number of recombinants observed should be regarded as proportional, but not equivalent, to the total number of zygotes formed.

The kinetics of segregation

Segregation is the process whereby the T+ L+ recombinant chromosome, which has already been formed within the diploid zygote, attains an independent position in a haploid cell. The situation is complicated by the fact that what is called a bacterial cell normally possesses two to four chromatinic bodies which are assumed, with reason, to be identical nuclear analogues (see Robinow, 1946). Hence when we refer to a zygote (i.e. a fertilized F- cell) we really connote a structure at the nuclear rather than at the cellular level, for if a part of only one Hfr chromosome enters the F- cell, as the usual clonal nature of recombinant colonies suggests, it presumably pairs with only one of a possible four of its F- homologues with the result that three of the nuclei of the fertilized F- cell will be haploid F- while the fourth will be diploid Hfr/F-. It is thus to be expected that the zygote cell, after recombination and segregation, will produce one or more F- cells in addition to whatever segregants the diploid nucleus may yield. This has been demonstrated experimentally by allowing freshly formed zygotes to segregate without selection on nutrient agar; every colony which was found by replica plating (Lederberg & Lederberg, 1952) to contain a prototrophic recombinant was also found to contain F- cells (Hayes, unpublished). With regard to the process of recombination, the main point of interest is the time at which that component of the cell containing the diploid nucleus divides to liberate a haploid recombinant. The interval between the formation of the zygote and its segregation defines, for future analysis, the vital period during which the process of recombination proper takes place.

The time at which the recombinant haploid cells issuing from the zygotes undergo their first and subsequent divisions, either on minimal agar or in broth, is easily ascertained. Young nutrient broth cultures of Hfr and F- cells are mixed and aerated at 37° for 30 min. to allow zygotes to form. The Hfr parental cells are destroyed by adding a high multiplicity of phage T₆ and aerating for 10 min. so that only zygotes and F- cells remain. Such a preparation is called a 'zygote suspension'. To assess the time of segregation on minimal agar, a series of minimal agar (+ vitamin B₁) plates, at 37°, is inoculated with diluted zygote suspension so as to yield, after incubation, about twenty to thirty prototroph colonies/plate, each colony being composed of the progeny of a T+ L+ recombinant segregant issuing from a single zygote. At intervals after inoculation and incubation, the surfaces of the plates, in turn, are vigorously rubbed with distilled water by means of a glass spreader. This separates the progeny of any T+ L+ recombinants that have already divided at the time of rubbing so that the subsequent colony count is doubled for each generation. Prior to division, of course, the colony count remains constant

since the effect of rubbing is simply to alter the position of the zygotes or segregants on the plate.

To study segregation in nutrient broth, the zygote suspension is diluted 1/50 into fresh broth at the desired temperature. Samples are removed as a function of time, appropriately diluted and plated on minimal agar to select for T+L+ recombinants. As before, every division is reflected in a doubling of the count of prototroph colonies.

The number of colonies obtained in such experiments remains static for a period and then, usually abruptly, commences to increase exponentially. The time at which this increase begins represents the time when the first division of the recombinant cells is initiated and is assumed to coincide with their first appearance as independent units (see Fig. 5, solid curves). Although we cannot be sure that this assumption is correct, it is at least certain that segregation has occurred at this time.

The kinetics of phenotypic expression

The Hfr genes determining resistance to sodium azide and to phage T₁ are closely linked to the selective markers T+L+ and are inherited respectively by about 90 and 75 % of T+L+ recombinants (see Fig. 1). For this reason, and because they manifest their effects in very different ways, these markers are well suited to the study of phenotypic expression. The experiments are run in parallel with those to determine the kinetics of segregation, either on minimal agar or in nutrient broth, but selection is made for the inheritance and expression of resistance, as well as for inheritance of prototrophy from the Hfr parent, by exposure to either sodium azide or phage T₁ at the time of sampling. Since, under the conditions employed, these agents prevent any further division of sensitive organisms, only those cells, whether zygotes or T+L+ segregants, which have inherited the gene controlling resistance, and in which the character of resistance has become expressed, can produce colonies. When the proportion of T+L+ recombinant colonies obtained in the presence of the drug reaches the proportion of T+L+ recombinants which have inherited the gene for resistance (i.e. *c.* 90 % for resistance to sodium azide and *c.* 75 % for phage T₁ resistance), expression is said to be complete.

Additional details of the experimental techniques used to demonstrate these methods will be found at the end of the next section.

MATERIALS AND TECHNIQUES

Bacterial strains

The great majority of the experiments to be described employed variants of two strains of *Escherichia coli* K-12: (1) The methionine-requiring (M-) strain, HfrH, isolated and described by Hayes (1953*b*); Hfr derivatives from this strain were occasionally used when alternative unselected markers were needed in the Hfr parent. Such derivatives were obtained either by recombination in crosses with a suitable F- parent (Hayes, 1953*b*) or by simple selection of

spontaneous mutants, and showed no variation from the parent strain with respect to the properties under investigation.

(2) An auxotrophic K-12F- strain called W-1 (Lederberg & Lederberg, 1952) which requires threonine, leucine and vitamin B₁ for growth (TLB₁-) and does not ferment lactose (Lac₁-) or maltose (Mal₁-). This strain was preferred to the classical TLB₁-F- strain, W-677, which was derived from it and which it closely resembles in its genetic markers, since some experiments demanded an F- parent sensitive to phage T₁ to which strain W-677 is resistant. The derivative of strain W-1 actually used was resistant to streptomycin (S^r) and to phages T₃ and T₆ (T₃^r, T₆^r), and was obtained from the parent strain in three steps by simple selection of spontaneous mutants.

Other strains occasionally employed will be specified in the text. All strains were lysogenic for λ phage.

Media

Minimal agar. The basic medium was that of Tatum & Lederberg (1947), solidified with 2.0% (w/v) powdered New Zealand agar which had been washed, by suspension in a muslin bag, in several changes of distilled water during 48 hr. The medium was supplemented before use with either glucose or lactose (0.25%, w/v) depending on the selective requirements, and, unless otherwise stated, with vitamin B₁ (thiamine) (5.0 μ g./ml.). Sodium aspartate was usually added to a final concentration of 0.1% (w/v) for reasons to be discussed later.

Nutrient broth. This was, in general, the tryptic digest of beef routinely employed by this Department. Latterly, however, a yeast extract + casein hydrolysate medium (0.5% Difco dehydrated 'Bacto' Yeast Extract + 2.0% Difco 'Bacto' Casamino Acids, Technical, in distilled water; pH adjusted to 7.4) was used.

Nutrient agar. This was made from the nutrient broth described above.

Buffer. An aqueous solution containing (% w/v): NaCl, 0.4; MgSO₄.7H₂O, 0.02; Na₂HPO₄, 0.7; KH₂PO₄, 0.3. The required pH value (usually 7.2) was obtained by addition of NaOH or HCl solution.

Basic technical procedures

Maintenance of stock cultures. Stock cultures were maintained at 4° on Dorset's egg medium in screw-capped bottles. Every 4-6 weeks, overnight broth cultures from the stock cultures were diluted and spread on blood agar plates so as to yield c. 50-100 colonies/plate. The plates were incubated overnight at 30° so that the colonies developed to only a small size, and were then stored at 4°.

Preparation of working cultures. The starting-point of most experiments was logarithmic phase nutrient broth cultures of the Hfr and F- parental strains. The evening before an experiment a single colony of each of the required strains was subcultured, from the blood agar plates maintained at 4°, to nutrient broth and incubated overnight at 37°. Cultures for use in the experiment were prepared by inoculating the desired volume of nutrient broth, warmed to 37°, with 1/10 vol. of the overnight broth culture and then aerating

at 37° for 1½ hr. Control experiments showed that these cultures were at about the middle point of the logarithmic phase of growth and had a viable count of $c. 2.5 \times 10^8$ organisms/ml.

Preparation of washed suspensions and mixtures. Unless otherwise stated, cultures were washed in three changes of buffer (pH 7.2) at room temperature. In most experiments involving comparison of the rates of zygote formation in different media or under different environmental conditions, washed suspensions of each parent were resuspended to 1/10 their original volume in buffer and 0.5 vol. of each were then added separately to 9.0 vol. of the test medium, so that the final population density was equivalent to that of a mixture of the original cultures ($c. 1.25 \times 10^8$ organisms/ml. of each parent).

Aeration. Cultures or suspensions were aerated in screw-capped bottles clipped flat to the periphery of a gramophone turntable, inclined at 45° to the horizontal and rotating at 33 r.p.m. in an incubator. The bottles were usually less than half full.

Viable counts. Appropriate dilutions of the suspension were made in buffer. Standard loopfuls were then transferred, by means of a welded platinum loop, to the surface of the agar medium and spread over areas of $c. 3$ cm. diam. Seven such areas can be accommodated on a plate of 9 cm. diam. The standard loop was calibrated by weighing and contained $c. 0.0125$ ml. The counts recorded here are, in general, the average of triplicates. When a higher degree of precision was required, counts were made in sextuplicate. Colonies were enumerated after overnight incubation, at 37° in the case of recombinant colonies on minimal agar, and at 30° for total viable counts on nutrient agar. Under these conditions the colonies, although easily visible without magnification, are small and as many as $c. 250/3$ cm. diam. area can be counted with accuracy.

Preparation of bacteriophage (T_6) suspensions. A suspension of the phage was added, at low multiplicity, to an exponentially growing nutrient broth culture ($c. 10^8$ viable organisms/ml.) of *Escherichia coli* strain B and aerated at 37° until clearing occurred. Residual viable bacteria were killed by heating at 58–60° for 20 min. in a water bath; further clarification by centrifugation was rarely required. Phage titres were usually 10^{10} – 10^{11} plaque-forming particles/ml. Phage T_6 , which was routinely used throughout this work, was the wild-type r^+ strain and had approximately the same efficiency of plating on *E. coli* K-12 strains as on *E. coli* strain B.

The techniques of kinetic analysis

Zygote formation. Suspensions of the two parents were mixed, usually in equal proportions, in a fluid medium warmed to the temperature at which the experiment was to be conducted. The mixture was aerated at that temperature. At intervals after mixing, 1.0 ml. samples were rapidly transferred to 1.0 ml. of phage T_6 suspension in nutrient broth, contained in screw-capped bottles of $c. 4.0$ ml. capacity. The mixture was shaken by hand for a few seconds and then aerated for 10–20 min. at 37°. In most experiments the multiplicity of phage T_6 infection was of the order of 50–100/IIfr (or F+) cell, giving a very

rapid rate of adsorption. The viable count of Hfr cells was reduced at least 1000-fold by the treatment. In $\text{Hfr} \times \text{F}^-$ crosses, the treated mixture was diluted in buffer and plated, without washing, on the selective medium. The extent of dilution required obviously depends on the experimental conditions: in media allowing optimal zygote formation, a final dilution of 10^{-3} to 10^{-4} was usually used. The amount of nutrient broth carried over to minimal medium in inocula from 10^{-2} dilutions was insufficient to allow visible growth of auxotrophic strains. In the case of $\text{F}^+ \times \text{F}^-$ crosses the treated mixture was washed 2-3 times, resuspended to (usually) $\frac{1}{4}-\frac{1}{2}$ vol. in buffer and plated without dilution. Three washings are required when selection is made for inheritance from the F^+ parent of the ability to synthesize vitamin B_1 . Using this technique, samples taken immediately after mixing never yielded any recombinant colonies.

Segregation. All the relevant information has already been given under 'Development of Methods'.

Phenotypic expression. To study expression on minimal agar, separate 3 cm. Petri dishes, containing 2.0 ml. minimal agar (+vitamin B_1 + 0.1 % sodium aspartate), were used for each plating. When the expression of sodium azide resistance was being assessed, the amount of sodium azide rubbed over the surface, in place of distilled water, was 0.025 ml. of an M/20 solution, giving a final concentration in the agar, after diffusion, of M/1600. This concentration was found to be optimally differential. In nutrient agar M/500 sodium azide is recommended for distinguishing resistant from sensitive organisms (Lederberg, 1950), but this concentration in minimal agar inhibits the growth of resistant prototrophs. In the phage T_1 experiments, 0.025 ml. of a washed phage suspension, containing 10^{12} plaque-forming particles/ml., was used.

To study expression in nutrient broth, the samples were spread on each of two series of minimal agar (+vitamin B_1 + 0.1 % sodium aspartate) plates, one of which was not supplemented further and was used to assess the kinetics of segregation; plates of the other series either contained M/1600 sodium azide, or had been preseeded with 0.1 ml. of the high-titre, washed, phage T_1 suspension.

RESULTS

Analysis of zygote formation in $\text{Hfr} \times \text{F}^-$ crosses

Young broth cultures of T_6^a .Hfr and T_6^a . F^- strains, containing approximately equal numbers of cells, were mixed and aerated at 37° . Samples were withdrawn at intervals, treated with phage T_6 and plated for $\text{T}^+ \text{L}^+$ recombinants. A typical curve relating the number of recombinants to the interval of time after mixing is given in Fig. 2. Unlike the curves obtained by Nelson (1951) with $\text{F}^+ \times \text{F}^-$ crosses, which arose from the origin, a lag of 8-10 min. regularly preceded the linear rise in the number of recombinants. Assuming a similar basic mechanism of mating in the two varieties of cross, this lag must result from the killing of the Hfr cells by phage T_6 and therefore represents the time required for transfer of the genes $\text{T}^+ \text{L}^+$ from the Hfr to the F^- cell; i.e. the time required for zygote formation. Between 30 and 40 min.

after mixing the slope of the curve begins to flatten, presumably due to the decreasing probability of fresh contacts arising between unpaired Hfr and F⁻ cells. Analysis of T⁺ L⁺ recombinants derived from samples treated with phage about 45 min. after mixing showed the same percentage inheritance of unselected markers from the Hfr parent as is usual in crosses made *ab initio* on minimal agar. At 60 min. after mixing, the number of recombinants formed

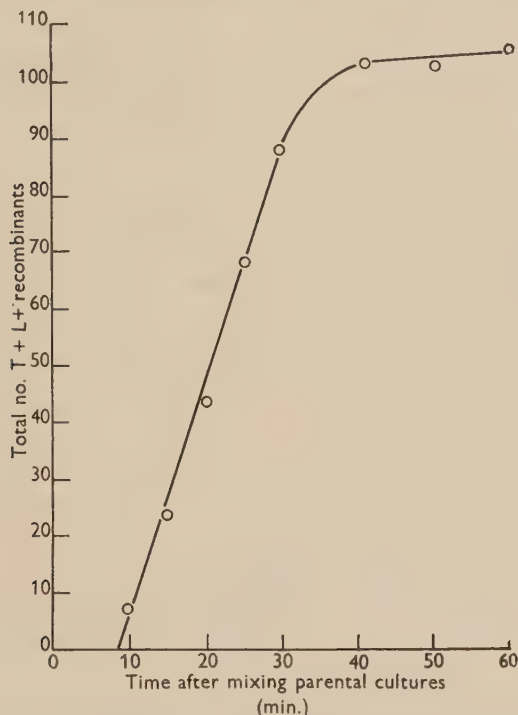


Fig. 2. The kinetics of zygote formation. Logarithmic phase broth cultures of HfrH.M-T₆⁺ and F⁻.TLB₁-T₆⁻ were mixed and aerated at 37°. Samples removed at intervals were treated with phage T₆, diluted, and plated to select the growth of T⁺ L⁺ recombinants. Thus the term 'zygote' here connotes an F⁻ cell which, at the time of treatment, had already received the genes T⁺ L⁺ from an Hfr cell.

was usually about 10% the number of viable F⁻ cells initially present. Exceptionally the ratio of T⁺ L⁺ recombinants to F⁻ cells was as high as 1:3 to 1:5. Decreasing 20-fold the number of F⁻ cells in the mixture, so that the Hfr cells were in gross excess, did not significantly raise the ratio nor alter the slope of the curve.

Physiological and environmental requirements for zygote formation

Nutritional requirements. About the time that the phage method for studying zygote formation was being evolved, it was observed that when thoroughly washed Hfr (or F⁺) and F⁻ cells were plated together on minimal agar prepared with highly purified agar (see Fisher, 1957*a*) virtually no recombinants developed. When, however, the agar was supplemented with

asparagine (Gray & Tatum, 1944) or, preferably, with sodium aspartate, the expected number of recombinants arose. The minimal effective concentration of sodium aspartate was about 0.01 % (w/v). Sodium pyruvate, succinate, malate or fumarate replaced aspartate, though much less efficiently, but the addition of amino acids other than those required for the growth of the parental strains was ineffective. Further investigation revealed:

(1) Prototrophic recombinants derived from a cross on highly purified minimal agar, supplemented with sodium aspartate, grew well, though rather more slowly, when subcultured to minimal agar without aspartate.

(2) Parental mixtures aerated together for 30 min. in broth, treated with phage to kill the donor cells and thoroughly washed as before (i.e. washed zygote suspensions), yielded the same number of recombinant colonies whether sodium aspartate was present or not.

These facts suggested that the stage of mating which was suppressed on purified minimal agar was that of zygote formation, and that the effect of aspartate might be associated with the operation of the Krebs cycle in the mating cells. The phenomenon was therefore investigated quantitatively by means of the phage method. When the parental cultures were washed and mixed in unsupplemented buffer, the number of zygotes formed at 30–45 min. after mixing was usually less than 1 % of the number arising from the same mixture in nutrient broth. Addition to the buffer of either glucose (0.25 %) or sodium aspartate (0.1 %) alone did not appreciably increase the yield of zygotes. When both glucose and sodium aspartate were added, however, the number of zygotes rose to about the same level as that found in broth. When the kinetics of zygote formation was studied in this medium, curves started to rise at 8–10 min. after mixing and began to flatten 20–30 min. later. The recent work of Fisher (1957*a*) has confirmed and greatly extended these findings.

A defined fluid medium which permits optimal zygote formation while restricting the multiplication of both parents is more suitable for the precise analysis of the requirements of this stage of mating than is nutrient broth. The following experiments were therefore carried out in buffer, usually at pH 7.2, supplemented with glucose (0.25 %) and sodium aspartate (0.02 %).

Cellular multiplication and zygote formation. In order to determine whether zygote formation could take place in the complete absence of cellular multiplication, washed parental suspensions were aerated for 30–45 min. in buffer + glucose + aspartate, both separately and after mixing. The number of F-cells (TLB₁ -) usually remained constant during this period, but the count of Hfr cells (M -) increased by as much as 25 %. It was suggested by Mr K. W. Fisher that this multiplication of Hfr cells might be due to their endogenous food reserves. The experiments were therefore repeated using washed parental suspensions which were starved, by aeration in unsupplemented buffer at 37° for 30 min., before being mixed in buffer + glucose + aspartate. Under these conditions the rate of zygote formation was only slightly decreased while no increase in the numbers of either parent could be detected by viable counts or nephelometry.

Washed suspensions mixed in unsupplemented buffer usually yield less than 1 % the number of zygotes formed by the same mixtures in buffer + glucose + aspartate. When the suspensions were starved to exhaust their endogenous reserves before mixing in unsupplemented buffer, the yield of zygotes became negligible. Zygote formation, therefore, does not require the multiplication of either parent but is strictly dependent on the presence of glucose and sodium aspartate when other nutrients are absent from the medium (see Fisher, 1957 *a, b*).

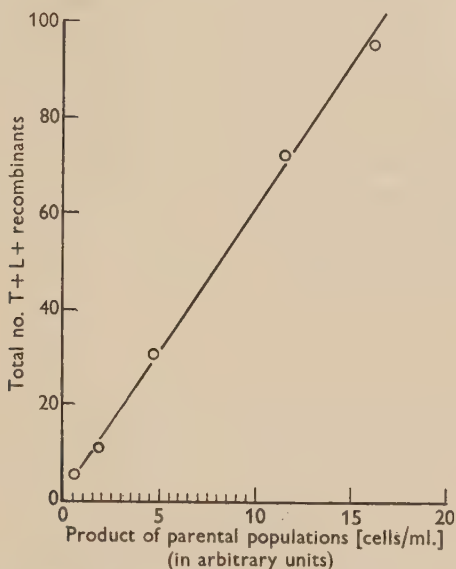


Fig. 3. The effect of parental population density on the number of zygotes formed. Cross = HfrH.M - T_6^+ \times F - .TLB₁ - T_6^+ in buffer + glucose + sodium aspartate (pH 6.5) at 37°. A series of dilutions of each parental suspension, differing by 25 %, were made in the test medium. Equal vols. of equivalent dilutions of each parental suspension were mixed and aerated at 37°; 35 min. later each mixture was treated with phage T_6 , appropriately diluted, and plated to select the growth of T + L + recombinants. The arbitrary unit was defined as the product of the no. cells/ml. of each parent in the most dilute mixture yielding a significant no. of recombinants (i.e. Hfr: $2.2 \times 10^7 \times$ F - : 2.0×10^7).

Effect of parental population densities. Washed parental cultures were suspended separately in buffer + glucose + aspartate (pH 6.5) at 37° to give *c.* 10^8 organisms/ml. A series of dilutions of each suspension was then made in the same medium in such a way that each successive dilution contained 25 % fewer cells than the previous one. Equivalent dilutions of each parent were mixed in equal parts and aerated at 37° for 35 min. Pilot experiments had shown that at this time the number of zygotes in the undiluted mixture was approaching a maximum. The mixtures at every dilution were then treated with phage T_6 , suitably diluted and plated for T + L + recombinants. The yield of recombinants from each dilution was found to be proportional to the product of the population densities of each parent (cells/ml.) as shown in Fig. 3. In conformity with the conclusions of Nelson (1951) in the case of

F + \times F - crosses, this finding, together with the initial linearity of the rate of zygote formation (Fig. 2), shows that the frequency with which zygotes are formed in Hfr \times F - systems is a function of the probability of collision between two parental cells.

Effect of temperature on zygote formation. Equal parts of washed parental suspensions were mixed in buffer + glucose + aspartate at 44°, 37°, 32°, 25° and 4°. After incubation at these temperatures for 45 min., each mixture was treated with phage T₆ and plated for T + L + recombinants as before. The numbers of recombinants formed, expressed as a percentage of those formed at 37°, was: 25 % at 44°, 80 % at 32°, 6 % at 25° and less than 1 % at 4°. Zygote formation is thus a temperature-dependent process and its efficiency falls rapidly as the temperature diverges from the optimal at 37°.

Effect of deoxyribonuclease (DNA-ase) on zygote formation. Equal portions of washed parental suspensions were mixed in buffer + glucose + aspartate + MgCl₂ (M/200), with and without DNA-ase (200 μ g./ml.), at 37°. The mixtures were aerated at 37° for 30 min. and then treated with phage T₆ and plated as before. The presence of DNA-ase had no effect on the efficiency of zygote formation (cf. Lederberg, 1947).

The kinetics of chromosomal transfer

Wollman & Jacob (1955), in their study of the kinetics of zygote formation in the HfrH \times F - cross, treated samples from the mating mixture in a Waring blender, instead of with virulent phage, in order to separate the mating cells. When untreated samples of the mixture were plated and the number of T + L + recombinants scored as a function of time, a curve was obtained which commenced to rise linearly from the time of mixing, began to flatten about 30 min. later and reached a plateau at about 50 min. This curve is similar to those obtained by Nelson (1951) from F + \times F - crosses and describes the kinetics of the formation of effective unions between Hfr and F - cells. On the other hand, curves obtained from the same samples after treatment in the blender showed a lag of 8-10 min. as in the kinetic experiments using phage, and thereafter rose to join the first curve at about 50 min. after mixing. Wollman & Jacob then scored the percentage inheritance of various unselected Hfr markers among the T + L + recombinants obtained at intervals after mixing (see Fig. 1). In the case of the untreated samples the unselected markers appeared in their normal frequencies irrespective of the time of sampling. In the treated series, however, the proportion of unselected Hfr markers steadily increased among the T + L + recombinants from successive samples, appearing in a definite sequence which corresponded to the order in which the genes are arranged on the chromosome as determined by genetic analysis. Thus the Hfr character T₁ was first found in T + L + recombinants within a few minutes of their appearance; but the first appearance of Lac + was delayed until 18 min., and of Gal + until about 28 min. after the parental suspensions were mixed. These results indicated (Wollman & Jacob, 1955) that the chromosome of the donor strain, HfrH, always enters the F - cell by an extremity, O, which is followed by the genes T + L + 8 min. later and then by the other loci at

intervals of time proportional to their distance from O on the chromosome. The transfer of the entire chromosomal segment O—Gal occupies roughly 30 min. The effect of treatment in the blender is to separate the mating cells and thus to break the chromosome during its transfer so that only that fragment which has already entered the F— cell at the time of treatment can participate in subsequent recombination.

These important findings of Wollman & Jacob have been fully confirmed by using the phage method to kill the Hfr parental cells, instead of separating the mating pairs by agitation. For example, at 37°, the times of entry into the F— cell of the Hfr loci T + L + and Lac + were found to be 8–10 and 17–20 min. respectively after mixing, whether the mixture was made in nutrient broth or in buffer + glucose + aspartate (see Fig. 4A).

It thus turns out that the kinetics of zygote formation only become meaningful when expressed in terms of the particular marker or markers transferred to the F— cell. For example, at about 8 min. after mixing an Hfr and an F— culture, zygotes which can segregate a T + L + recombinant begin to be formed; if, however, selection is made for zygotes which can segregate T + L + Lac + recombinants, it is found that such zygotes do not appear until 18 min. after mixing, since the Lac + gene does not begin to enter the F— cells, which have already received T + L +, until this time.

*The influence of environmental factors on the kinetics of
chromosomal transfer*

Fisher (1957*a, b*) has shown that energy is required for zygote formation to enable the Hfr cell to convert a chance collision with an F— cell into an effective union, and thereafter to inject its chromosomal contribution. The dependence of zygote formation upon temperature is due to this requirement for energy. There are also factors influencing zygote formation which do not involve energy-dependent processes. Among these is population density which determines the frequency of chance collisions, and the differences in surface structure, described by Maccacaro (1955) and Maccacaro & Comolli (1956) as distinguishing F + from F— cells, which may well be decisive in permitting chance collisions to become effective unions.

Experiments were devised to analyse the effect of these various factors on the form of the curves describing the kinetics of zygote formation and chromosome transfer in Hfr × F— crosses.

Effect of energy restriction. Diminution of temperature was used to lower the overall capacity of the Hfr cells to produce energy by oxidation of carbohydrate. Two equivalent mixtures of washed Hfr and F— cells were made in buffer + glucose + aspartate (pH 7.2), one at 37° and the other at 32°; these mixtures were then aerated under standard conditions at 37° and 32°, respectively. Samples were removed at intervals, treated with phage T₆ and plated on each of two minimal media, one selecting for all recombinants inheriting the Hfr genes T + L +, the other for recombinants inheriting both T + L + and Lac +. Results are shown in Fig. 4A. At 37°, the genes T + L + (continuous line) begin to enter the F— cells at 8 min. and the gene Lac +

(interrupted line) at 17 min. after mixing, so that transfer of the piece of chromosome from TL to Lac takes 9 min. at this temperature. At 32°, however, T+L+ enters at 18 min. and Lac+ at 38 min., so that transfer of the same piece of chromosome takes just twice as long at the lower temperature. Since effective unions between Hfr and F- cells are formed very rapidly (see above), it may be assumed that transfer of the O—TL segment of chromosome occupies virtually the whole of the 8 min. lag period which precedes entry of the T+L+ genes into the F- cells. At 37°, therefore, transfer of the piece

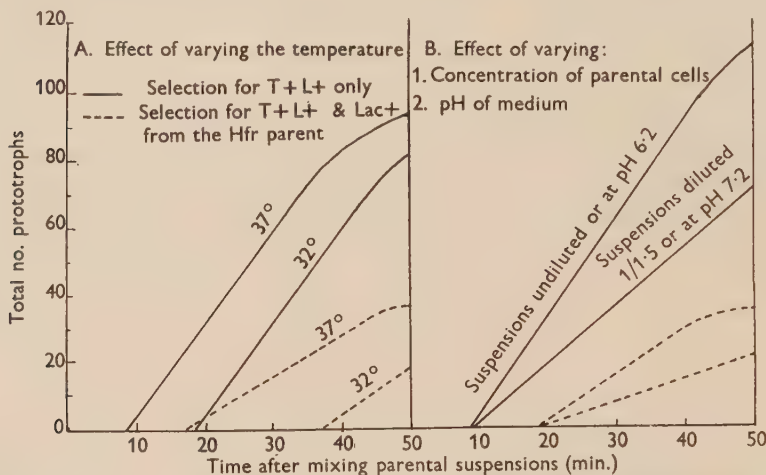


Fig. 4. The effect of environmental factors on the kinetics of zygote formation and chromosomal transfer. Cross = HfrH.M-T₆^s × F-.TLB₁-T₆^r in buffer+glucose+sodium aspartate. At intervals after mixing, samples were treated with phage T₆, diluted and plated on:

- Minimal agar+vitamin B₁+glucose (selection for inheritance of T+L+ only from the Hfr parent).
- Minimal agar+vitamin B₁+lactose (selection for inheritance of T+L+ and Lac+).

(A) Shows the effect of varying the temperature at which zygote formation occurs.

(B) Shows the effect of varying the concentration of the parental cells, or the pH value of the medium.

of chromosome O—TL takes 8 min. and of the piece O—TL—Lac 17 min.; at 32°, transfer of these same pieces takes 18 and 38 min. respectively. The proportionality between these times strongly suggests that that part of the chromosome between O and Lac, at least, enters the F- cell at a uniform rate which depends exclusively upon the available energy. The fact that the comparable curves of Fig. 4A are parallel, although displaced on the time axis, indicates that the only effect of this degree of limitation of energy is to slow the rate of chromosome transfer.

Effect of varying the frequency of chance collisions. Washed cultures of each parent were suspended separately in buffer+glucose+aspartate (pH 7.2) at 37°. A portion of each suspension was then diluted 1/1.5 in the same medium. Equal volumes of the two parental suspensions at each concentration were

mixed, aerated at 37° and sampled at intervals as before. Results are shown in Fig. 4B. The times of entry of the genes $T+L+$ and $Lac+$ into the $F-$ cells remain unaltered when the population density of the parental cells is reduced. In each case the curves arise from the same point on the time axis but their slopes differ.

Fisher (1957*b*) has shown that lowering the pH value of the medium from 7.2 to 6.2 more than doubles the number of zygotes formed. When the kinetics of chromosomal transfer were compared for identical $Hfr \times F-$ crosses in buffer + glucose + aspartate at these two pH's, the same kind of result was obtained as when the population density was varied (see Fig. 4B). Since the population density was the same in both crosses, lowering the pH value presumably exercises a surface effect, probably ascribable to an alteration of charge, which increases the proportion of random collisions which can subsequently become effective unions (Maccacaro & Comolli, 1956).

The kinetics of zygote formation in $F+ \times F-$ crosses

The blender technique is not well adapted to study of the kinetics of $F+ \times F-$ crosses since the high concentrations of parental mixtures which must be plated, after treatment, to give significant prototroph counts will lead to secondary contacts, followed by the occurrence of recombination, on the plate. On the other hand, the phage method, in which the donor parental population is reduced to an insignificant level before plating, is well suited to such an investigation.

Young nutrient broth cultures of the $M-F+$ strain 58-161 (genotypically identical with strain $HfrH$) and the TLB_1-F- strain previously used (or the similar strain, W677. T_6') were mixed and aerated at 37°. Samples were removed at intervals, treated with phage T_6 , washed, resuspended in the appropriate volume of buffer and finally plated on minimal agar supplemented with:

- (1) Glucose + vitamin B_1 (i.e. selection for inheritance of $T+L+$ only).
- (2) Lactose + vitamin B_1 (i.e. selection for inheritance of $T+L+$ and $Lac+$ from the $F+$ parent).
- (3) Glucose only (i.e. selection for inheritance of $T+L+$ and B_1+ from the $F+$ parent).

The times at which the $F+$ markers $T+L+$ and $Lac+$ began to appear were the same as in the equivalent Hfr cross (i.e. *c.* 8 and 18 min. after mixing respectively). The $F+$ marker B_1+ began to appear among recombinants about 45 min. after mixing and rose to its usual level of inheritance (*c.* 5.0 %) at about 60 min. When selection was made for the $F+$ markers $Xyl+$, $Mál+$ or S^r (streptomycin resistance) in addition to $T+L+$ (see Fig. 1) no recombinants were found (i.e. less than 0.5 % of those formed when only $T+L+$ was selected) up to 90 min. after mixing.

The inheritance among $T+L+$ recombinants of markers from the donor parent is therefore very much the same whether the cross is made by the classical method of mixing the parental suspensions *ab initio* on minimal agar, or by the method here described of permitting the zygotes to form under

controlled conditions in fluid medium before plating, provided that in the latter method adequate time is allowed for chromosomal transfer before the mating process is arrested with phage (or agitation). The two methods have, however, been found to yield significant quantitative differences in two respects:

(1) When direct plating is used, the frequency with which $T+L+$ recombinants appear in the $Hfr \times F-$ cross is $c. 10^3$ times higher than in the equivalent $F+ \times F-$ cross (Hayes, 1953*b*). Yet when the two crosses are made in aerated broth under the same conditions of population density, and sampled when the number of zygotes has reached its peak, the Hfr cross yields $c. 2 \times 10^4$ times as many prototrophs as the $F+$ cross.

(2) When an $M-Hfr \times TLB_1-F-$ cross is made by direct plating on minimal agar devoid of vitamin B_1 so that the gene B_1+ as well as $T+L+$ must be inherited from the Hfr parent to form a prototroph, the recombination rate is $c. 10^3$ times less than when the same cross is made on minimal agar supplemented with vitamin B_1 . This means that B_1+ is only inherited by $c. 0.1\%$ of the $T+L+$ recombinants formed at high frequency as compared with $c. 5.0\%$ in the equivalent $F+ \times F-$ cross (see Hayes, 1953*b*). Since, however, the recombination rate of the $F+ \times F-$ cross is $c. 10^3$ times lower than that of the $Hfr \times F-$ cross when selection is made for $T+L+$ only, a simple calculation will show that, cell for cell, the Hfr cross yields about 20 times more B_1+ prototrophs than does the $F+$ cross. Nevertheless, when $Hfr \times F-$ zygotes are formed in aerated broth, no $T+L+B_1+$ recombinants have ever been isolated from samples taken up to 90 min. after mixing, although the technique is sensitive enough to detect one B_1+ prototroph in 5×10^5 .

The kinetics of segregation

Experiments to determine the times of segregation and of phenotypic expression in $Hfr \times F-$ crosses, both on minimal agar and in nutrient broth, have given very constant results. The kinetics of segregation is exemplified by the curves (continuous line) shown in Fig. 5. On minimal agar at 37° (Fig. 5A), $T+L+$ recombinants begin to divide about 120 min. after plating (i.e. 160 min. after mixing the parental cultures) and thereafter multiply with a generation time of about 60 min. In aerated broth at 37° (Fig. 5B), division of the recombinant segregants commences at about 100 min. after dilution of the zygote suspension into fresh broth (i.e. about 140 min. after mixing the parental cultures), and the generation time is 20 min.

Two difficulties arise when one tries to infer the actual time of segregation from these findings. First, when zygotes are made in broth (as described above) and aerated without dilution, it is found that no increase in the number of $T+L+$ recombinants occurs for at least 6 hr., whereas division begins 100 min. after dilution into fresh broth. Furthermore, there appears to be a tendency for the early divisions of the segregants to occur in bursts. This suggests that events in the zygotes achieve a measure of synchrony through inhibition of some step preceding segregation, which is released by dilution. The mechanism of this inhibition has not yet been investigated. It poses the

question of whether the timing of segregation should start when the parental cultures are mixed, or when the zygote suspension is diluted. This dilemma may assume importance when it becomes possible to attempt to correlate segregation with events occurring within the zygote during recombination.

The second difficulty is that although the time can be fixed at which $T + L +$ segregants first begin to divide, we cannot be sure that this coincides with the first appearance of the recombinant cells as independent units. For example, physiological delay in the expression of the selective markers used could introduce a lag between segregation and initiation of the first division; the relatively similar periods required for the first division in broth and on minimal agar, in contrast to the threefold increase in generation time on the latter medium (see Fig. 5A, B), make such a lag improbable when $T + L +$ is selected.

The absence of non-recombinant donor cells among zygote progeny

Since the discovery of one-way genetic transfer in *Escherichia coli* there has been controversy whether the absence among recombinants of certain markers of the donor parent is due to transfer of only part of the donor chromosome to the F^- cell to form an incomplete zygote (pre-zygotic elimination; Hayes, 1953*a, b*) or to elimination from a complete zygote of part of the donor contribution, either during or after recombination (post-zygotic elimination). The latter hypothesis is supported by diploid analysis (Nelson & Lederberg, 1954). If zygotes from the $HfrH \times F^-$ cross really receive the complete Hfr chromosome, then a proportion of them should segregate an Hfr parental cell, especially if recombination occurs at the 4-strand stage. The $HfrH$ and F^- strains used in the present work yield colonies of such different appearance, when examined by oblique illumination, that Hfr sectors can clearly be observed in F^- colonies when the two strains are mixed together and plated on nutrient agar without phage treatment. Thus segregation of an Hfr cell from a zygote should reveal itself as a sector, since colonies arising from zygotes are nearly always similar in appearance to F^- colonies. In the course of performing viable counts on parental mixtures, after zygote formation and destruction of the Hfr parent by phage, many thousands of colonies on nutrient agar have been examined, including many hundreds derived from zygote segregation. Only once was an Hfr sector observed, and since a recombinant could not be isolated from this colony there is no evidence that it was indeed derived from a zygote and that the sector did not arise accidentally from an Hfr contaminant which had survived the phage treatment.

The kinetics of phenotypic expression

When the kinetics of phenotypic expression of either sodium azide or phage T_1 resistance was plotted in terms of the generation time of the recombinants, closely similar results were obtained on minimal agar, nutrient agar and in nutrient broth. The patterns of expression of these two markers are quite different. Expression of the character Az^r begins at the time of dilution (or of plating) of the zygotes and then rises exponentially to become complete

just before the recombinants which inherit it start to divide (Fig. 5B: interrupted line). In contrast, the character T_1^r does not begin to be expressed until after segregation while full expression (i.e. in 75% of all $T+L+$ recombinants) is delayed until the 4th recombinant generation (Fig. 5A: interrupted line). Work on *Escherichia coli* K-12 diploid strains has shown that the gene T_1^r is recessive to T_1^s (Lederberg, 1949) so that delay in its expression until after segregation has occurred is to be expected; the fact

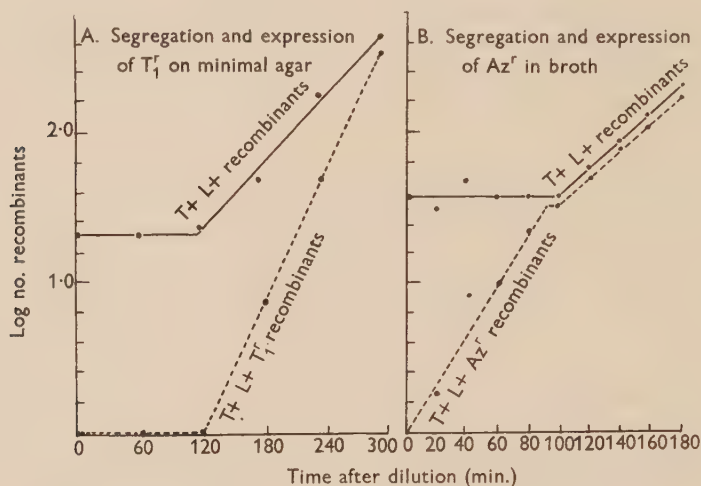


Fig. 5. The kinetics of segregation and phenotypic expression.

Cross = $HfrH.M - Az^rT_1^rT_6^s \times F - .TLB_1 - Az^sT_1^sT_6^s$.

The methods and techniques employed are described in the text.

that four generations are required before expression is manifest in all the cells which have inherited the gene, is probably a consequence of the dependence of phage resistance on structural changes in the cell wall. Since expression of the character Az^r increases rapidly during a period when the genes Az^r from the donor parent and Az^s from the recipient parent must be present together in the diploid zygote, it follows that Az^r is dominant to Az^s .

DISCUSSION

This study, which has been so notably extended by the recent work of Wollman & Jacob, and of Fisher, has attempted to define and analyse experimentally some of the presumptive stages of mating in *Escherichia coli* K-12. Since a comprehensive account of present knowledge about the mating process will be published elsewhere (Wollman *et al.* 1956), only a few points specifically raised by this paper will be discussed. The conclusion of Wollman & Jacob (1955), that the observed effects of treating mating cells in a blender are due to breakage of the Hfr chromosome during its transit into the $F-$ cell, has been criticized on the ground that similar effects might result from violent agitation of fully formed zygotes at different stages of pairing or recombination (Lederberg,

1955). This objection also applies to the use of 2, 4-dinitrophenol to arrest genetic transfer (Fisher, 1957*b*) since this agent interferes with the energy utilization of the recipient as well as of the donor cells. Since precisely similar effects are observed to follow selective killing of the donor parent by the virulent phage T_6 , the validity of this objection is greatly weakened. The recipient cells are resistant and do not adsorb the phage which, therefore, could only influence the course of pairing or recombination if its DNA entered the zygote by the same route as the donor genetic material. Although the rare occurrence of such an event has not been excluded it is likely that its outcome, if it did occur, would be death of the zygote from the multiplication of phage within it. The number of recombinants found, and thus the number of zygotes formed, is not decreased by the use of phage. Moreover, Jacob & Wollman (1954) showed that the virulent phage, λ_2 , is not transferred in this way when sensitive HfrH cells mating with resistant F⁻ cells are superinfected with it.

Repeated attempts to obtain kinetic curves, with untreated mixtures, which did not show a lag were unsuccessful until the rather vigorous dilution technique used was replaced by a very gentle one. In fact, the only really reliable method is initially to dilute the samples very gently to 10^{-2} in buffer + glucose + sodium aspartate at 37°, in order to prevent further contacts, and then to incubate the diluted mixture for 10 min. before making the final dilution for plating. The short period of incubation allows the Hfr selective markers, T + L +, to enter the F⁻ partner of all the pairs which had been formed at the time of the initial dilution, so that subsequent separation of these pairs by further dilution and plating does not lower the recombinant count. An intensity of agitation very much milder than that imposed by a blender is thus adequate to produce the Wollman-Jacob effect. It should be stressed that the sensitivity of the rate of zygote formation to small changes in temperature, population density and pH value makes the strict control of these factors vital in comparative studies.

Jacob & Wollman (1956) have produced evidence that the fertility of F⁺ × F⁻ crosses is due solely to the occurrence of Hfr mutants in the F⁺ population. When they analysed crosses involving some of these Hfr mutant strains by the blender method, they found not only that different Hfr strains might transfer different segments of the donor chromosome to the F⁻ cells at high frequency, but that the order in which the same genes were transferred might differ from strain to strain. There is thus evidence that mutation to the Hfr state may be associated with chromosomal rearrangements. Application of the phage method to the kinetics of chromosomal transfer in an F⁺ × F⁻ cross, analogous in genotype and polarity of transfer to the standard HfrH × F⁻ cross, has revealed an identity of the two systems with respect to the timing and the order of entry of the genes T +, L + and Lac +. This suggests that the great majority of Hfr mutants (of the F⁺ strain used) which can transfer these markers are similar to the standard HfrH strain. However, the inheritance of the F⁺ marker B₁ + among T + L + recombinants with a frequency of 5%, and its entry into the F⁻ cell at 45 min., implies that at least 5% of Hfr mutants which transfer T + L + also transfer B₁ + at high

frequency, and that a greater length of chromosome than usual is involved. The marker B_1+ is not transferred to the zygote by strain HfrH.

The difference between the kinetics of phenotypic expression of sodium azide and phage T_1 resistance is striking. The fact that resistance to sodium azide is expressed in zygotes which are heterozygous for this character shows that resistance must be dominant to sensitivity to this drug. This is an exception to the rule that wild-type alleles are dominant to their mutant alleles in *Escherichia coli*. On the other hand, the dominance of sensitivity to phage T_1 is confirmed by the fact that resistance to this agent is not expressed until the recombinants begin to divide. This coincidence in timing also suggests that there is no lag between segregation and the initiation of division of the haploid segregants. The exponential nature of the rise in phenotypic expression of both the characters studied may reflect the fact that both are, basically, the result of enzyme synthesis.

Knowledge of the kinetics of phenotypic expression following recombination in bacteria is important for two reasons. First, it serves as a control against which the results of mutation kinetics can be studied. Secondly, it is necessary for the rational design of recombination experiments, whether in *Escherichia coli* or in other bacterial species. For example, the choice of selective markers which are recessive, or whose expression is manifested slowly, is unlikely to yield recombinants from an otherwise fertile cross unless application of the selective agent is withheld until expression has occurred. This is clearly demonstrated by the fact that virtually no recombinants appear if recently formed zygotes, which have inherited from the donor parent the gene determining resistance to sodium azide, are plated directly on to media containing the drug, although resistance is dominant to sensitivity and becomes rapidly expressed.

I wish to express my appreciation of the hospitality and kindness of Professor Max Delbrück, and of the many helpful discussions I enjoyed with him and his colleagues at the California Institute of Technology. I am also much indebted to Professor Boris Ephrussi, in whose laboratory part of the work was done, for his interest in this research, and to the British Empire Cancer Campaign for a grant in support of it. My thanks are also due to Dr J. C. White for a gift of deoxyribonuclease and to Dr G. Popjak for the use of a Spinco ultracentrifuge.

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The Role of the Krebs Cycle in Conjugation in *Escherichia coli* K-12

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SUMMARY: Zygote formation between strains of *Escherichia coli* K-12 is dependent on a supply of free energy made available by the oxidation of carbohydrate via the Krebs cycle reactions. The simultaneous addition of glucose and a dicarboxylic acid of the tricarboxylic acid cycle, or an immediate precursor, to mating cells markedly stimulates the number of zygotes formed. Reagents known to inhibit reactions of the Krebs cycle inhibit zygote formation. Evidence was obtained that synthesis of protein or either pentose or deoxypentose nucleic acid was not necessary for zygote formation.

Genetic recombination in *Escherichia coli*, strain K-12, was first demonstrated by the isolation of prototrophic clones from a mixture of two auxotrophic mutant strains having multiple and complementary nutritional requirements (Lederberg & Tatum, 1946). When the parental strains were differentially marked by other characters not involved in prototroph formation (e.g. carbohydrate fermentation, and resistance to drugs such as streptomycin or sodium azide and to bacteriophages), genetic analysis showed that these unselected markers were linked to one another as well as to the selected, auxotrophic markers (Lederberg, 1947). Despite this continuity of linkage, however, the genetic data were inconsistent with a completely linear arrangement of the genes on a single chromosome (Newcombe & Nyholm, 1950; Lederberg, Lederberg, Zinder & Lively, 1951; Rothfels, 1952).

The differentiation of fertile *Escherichia coli* strains into two mating types, one of which acted as a genetic donor and the other as a recipient, was first described by Hayes (1952). It was later shown that the donor character, called F (for fertility), could be transferred to recipient cells independently of the usual genetic markers and with an efficiency of the order of 100% (Lederberg, Cavalli & Lederberg, 1952; Cavalli, Lederberg & Lederberg, 1953; Hayes, 1953*a*). Crosses between recipient (F-) strains were sterile; crosses between donor (F+) and recipient strains showed maximum fertility. Thus F appeared as a factor which controlled the ability to transfer genetic material as well as the direction of transfer.

In F+ × F- crosses the maximum yield of recombinants is about 10⁻⁴ to 10⁻⁵ per F+ cell. Two donor mutants were independently isolated from strain 58-161 F+ which were about 1000 times more productive in crosses with the same F- strain (Cavalli, 1950; Hayes, 1953*b*). These mutants, which were unable to convert F- cells to the F+ state, were called Hfr (high frequency of recombination).

Unlike transformation and transduction, transfer of genetic material from

donor to recipient cell in *Escherichia coli* requires actual cellular contact and is, therefore, mediated by conjugation. The kinetics of this process in fluid media was first studied by Nelson (1951) who showed that it was analogous to a second-order reaction involving only single contacts between the participating cells. Hayes (1957) devised a method for studying the kinetics of zygote formation in Hfr \times F $-$ systems. Samples of a mixture of the two parents were removed at intervals after mixing and treated with virulent phage to which only the Hfr (donor) cells were susceptible. The donor cells were thus rapidly killed so that only those F $-$ recipient cells which had already been fertilized at the time of adding the phage (i.e. zygotes) could produce recombinants. Hayes had observed that when well-washed parental suspensions were plated together on minimal medium prepared with highly purified agar, no recombination occurred. The cross was normally productive, however, when the medium was supplemented with either asparagine or aspartic acid. In contrast, washed zygotes, which had already been formed in broth, produced the expected number of recombinants on the unsupplemented medium, which also supported the growth of recombinant prototrophs. It thus appeared that asparagine or aspartic acid were specifically required for zygote formation (Hayes, 1957). This paper describes an investigation into the physiological requirements for zygote formation which was initiated by these observations.

Definition of terms

(1) *Zygote*. This connotes a recipient (F $-$) cell which has received a chromosomal contribution from a donor (Hfr or F $+$) cell. Only a proportion of zygotes will yield recombinants of any given genetic constitution.

(2) *Recombination rate*. This is expressed as the number of recombinants of any given class per 100 F $-$ cells initially present.

METHODS

Organisms. Strain 58-161 F $+$ is a methionine-requiring (M $-$) *Escherichia coli* K-12 mutant sensitive to streptomycin (S s) and sodium azide (Az s). This strain ferments lactose and maltose (Lac $+$, Mal $+$), and is sensitive to coliphage T $_6$ (V $_6^s$).

Strain 58-161 Hfr, isolated by Hayes (1953*b*) carries the same nutritional markers as 58-161 F $+$. It is, however, resistant to streptomycin (S r) (250 μ g./ml.) and to sodium azide (Az r) (M/500).

Strain W-1 F $-$ (Lederberg & Lederberg, 1952) requires threonine, leucine and thiamine (B $_1$) for growth (TLB $_1-$). Its sugar fermentation markers are Lac $_1-$ and Mal $_1-$ and the variant used in this work was S r and V $_6^s$.

Strain W-677 F $-$ (Lederberg, 1950) also requires threonine, leucine and thiamine for growth (TLB $_1-$). The strain is sensitive to streptomycin and resistant to M/500 sodium azide (Az r).

Buffer. An aqueous solution containing 0.4% (w/v) NaCl, 0.02% (w/v) MgSO $_4 \cdot 7$ H $_2$ O and M/20 KH $_2$ PO $_4$, the pH value required being obtained with 0.2M-NaOH.

Minimal agar was as described by Tatum & Lederberg (1947) except that asparagine was omitted from the medium which was solidified with 2% (w/v) agar and supplemented with 0.2% (w/v) D-glucose and 5 μ g. thiamine/ml. (medium MAB₁). The agar powder was washed with distilled water for 2 days before use.

Nutrient broth was a beef digest broth routinely used in this department.

Chemical reagents. With two exceptions, the reagents were supplied by British Drug Houses Ltd and, except for DL-malic acid, were all of A.R. quality. Sodium monofluoroacetate was very kindly provided by Dr S. R. Elsdon and the L-aspartic acid was obtained from L. Light and Co., Colnbrook. Before use all stock solutions of the acids were adjusted to pH 7 with sodium hydroxide.

Coliphage T₆ suspensions. Cultures of *Escherichia coli* strain B, growing logarithmically in nutrient broth, were infected with phage T₆ at low multiplicity. The mixture was aerated until lysis occurred. Phage T₆ propagated in this manner normally yielded 10¹⁰ to 10¹¹ plaque-forming particles/ml. Residual *E. coli* cells were killed by heating at 56°–58° for 30 min.

Experiments to test the conditions necessary for zygote formation, unless otherwise specified, were carried out in 4 × $\frac{5}{8}$ in. test tubes sealed with rubber bungs. Between experiments the tubes were stored in chromic-sulphuric acid and the bungs in distilled water. Before use, both tubes and bungs were washed several times in distilled water and then autoclaved.

Basic experimental procedure

Strains were stored on Dorset egg slopes at 4°. Cultures for use were propagated from such stocks by overnight growth in nutrient broth, without aeration, at 37°. The following morning 1 vol. of each parental culture was diluted into 10 vol. of fresh broth at 37° and incubated at this temperature in screw-cap bottles for 105 min. on an inclined turntable rotating at 33 r.p.m. The volume of the bottles was such that an adequate supply of oxygen was available to the organisms during growth. At the end of the incubation period the cultures were washed separately with three changes of buffer (pH 7.2). The yield of organisms from 2.5 ml. of culture of each parent strain was then mixed and centrifuged. The supernatant fluid was removed and the mixed deposit resuspended in 5 ml. of warmed test medium and aerated at 37° for 30 min. on the turntable. At the end of this period 1 ml. of the mixture was added to an equal volume of coliphage T₆ suspension and aerated for 20 min. at 37°. This procedure destroyed the Hfr or F+ parent (since both are sensitive to T₆) and thus stopped further zygote formation. Such a treated mixture, in which only zygotes and F– cells remained viable, will be referred to as a zygote suspension.

Plating and counting techniques

Detection of zygotes. In the Hfr × F– and F+ × F– crosses used, prototrophs arise on MAB₁ as a result of selection for that recombinant class in which the closely linked genes T + L + inherited from the donor parent replace

the T-L- genes determining the auxotrophy of the recipient (F-) cells. Since zygotes of the same genetic constitution may yield different recombinant classes, and since not all zygotes do yield recombinants (see Wollman, Jacob & Hayes, 1956), it is clear that the total number of zygotes cannot be directly estimated. In order to study zygote formation, therefore, the assumption was made that the number of zygotes is directly proportional to the number of T+L+ prototrophic recombinants formed.

The number of T+L+ recombinants was determined by diluting the zygote suspension by a factor of 2×10^{-4} in buffer (pH 7.2). Samples (0.01 ml.) of the diluted suspension were then plated in triplicate on medium MAB₁, by using a standardized, welded platinum loop. The number of prototrophic colonies was counted after 18 hr. of incubation at 37°.

Total viable counts. These were carried out on the parental suspensions immediately before they were mixed. The suspension was diluted 10^{-5} in buffer (pH 7.2) and 0.01 ml. volumes were plated in triplicate on nutrient agar with a standardized platinum loop. Colonies were counted after 18 hr. of incubation at 30°.

RESULTS

Zygote formation on solid media

Washed suspensions of strains 58-161 Hfr and W-1 F- were plated together on:

- (1) minimal agar+thiamine prepared with unwashed agar (MAB₁);
 - (2) minimal agar+thiamine prepared with agar specially washed with sodium hypochlorite solution (5 % available chlorine), tap and distilled water, and acetone (WMAB₁);
 - (3) a similar minimal medium supplemented with thiamine and solidified with silica gel in place of agar (MSB₁);
 - (4) the same media supplemented with 0.025 % sodium L-aspartate.
- Prototrophs were counted after 18 hr. of incubation at 37°.

Table 1. *The formation of prototrophs on solid media*

Parent suspensions of 58-161 Hfr and W-1 F- were grown aerobically in nutrient broth, washed three times with buffer and resuspended in buffer (pH 7.2). Using a welded platinum loop, triplicate 0.01 ml. samples of each suspension were spread over the surface of 2.5 cm. plates of media. Counts were read after incubation at 37° for 18 hr.

Media*	Prototrophs (no. 1 plate)
MAB ₁	> 500
WMAB ₁	56
WMAB ₁ +0.025 % sodium L-aspartate	> 500
MSB ₁	0
MSB ₁ +0.025 % sodium L-aspartate	> 500

* MAB₁=minimal agar+glucose+thiamine. WMAB₁=minimal agar+glucose+thiamine, solidified with the specially washed agar (see text). MSB₁=minimal medium+glucose+thiamine, solidified with silica gel.

The results (Table 1) showed that purified agar or silica gel lacked some unknown substance which was essential for the formation of prototrophic

recombinants. This apparent deficiency was remedied by the addition of L-aspartic acid. Aqueous extracts of powdered agar were prepared and added to WMAB₁ but prototrophs were not formed even when concentrated (15 times) extracts were added. The same extracts were examined by paper chromatography for the presence of amino acids. Butanol/acetic acid/water (40:10:50) was used as the developing agent and the chromatograms were sprayed with 0.1% ninhydrin in water-saturated *n*-butanol. No amino acids were detected. The extracts were not examined for substances other than amino acids.

Zygote formation in fluid media

The results obtained by supplementing washed minimal agar with aspartic acid suggested that this substance was necessary for zygote formation. Zygotes which had been preformed in nutrient broth yielded recombinants with the same efficiency on medium WMAB₁ as on medium MAB₁. The role of aspartic acid in zygote formation in fluid media was therefore examined. In several experiments, the recombination rate of an Hfr × F⁻ mixture in unsupplemented buffer at pH 7.2 was consistently less than 3% of that observed in nutrient broth when samples were compared at 30 min. after mixing. A low rate was also observed when a mineral salt solution similar to that used in the preparation of minimal agar was substituted for the buffer. When the buffer was supplemented with aspartic acid alone, only a very small rise in the recombination rate was detected. Similarly, the addition to the buffer of glucose alone elicited only a slight increase in the rate. The simultaneous addition of both glucose and aspartate, however, yielded a number of T + L + recombinants equal to or exceeding the number obtained in nutrient broth. The results of these experiments are summarized in Fig. 1.

It was found that the recombination rate varied directly with the concentration of aspartic acid within the range 1–80 µg./ml. Concentrations below 1 µg./ml. had no detectable effect on the recombination rate, whilst those between 80 µg. and 200 µg./ml. did not produce a further increase in the number of zygotes formed (see Fig. 2). Concentrations of aspartic acid greater than 200 µg./ml. inhibited zygote formation.

The role of aspartic acid in zygote formation

It was thus established that aspartic acid and glucose are required for zygote formation. How do these substances act? Three possible modes of action of the aspartic acid were considered: (1) its possible importance as an essential intermediate of a biosynthetic pathway; (2) as an adsorption co-factor enabling the parent cells to make effective contact; (3) as a means of entry to the Krebs cycle for a C₄ fragment which would stimulate the oxidation of C₂ fragments (derived from glucose) via the tricarboxylic acid cycle.

The demonstration in *Escherichia coli* of the reversible reaction aspartic acid ⇌ fumaric acid + ammonia (Quastel & Woolf, 1926), as well as the possession by this organism of a glutamic-aspartic transaminase (Lichstein & Cohen, 1944) rendered the first possibility feasible since these two systems, linked

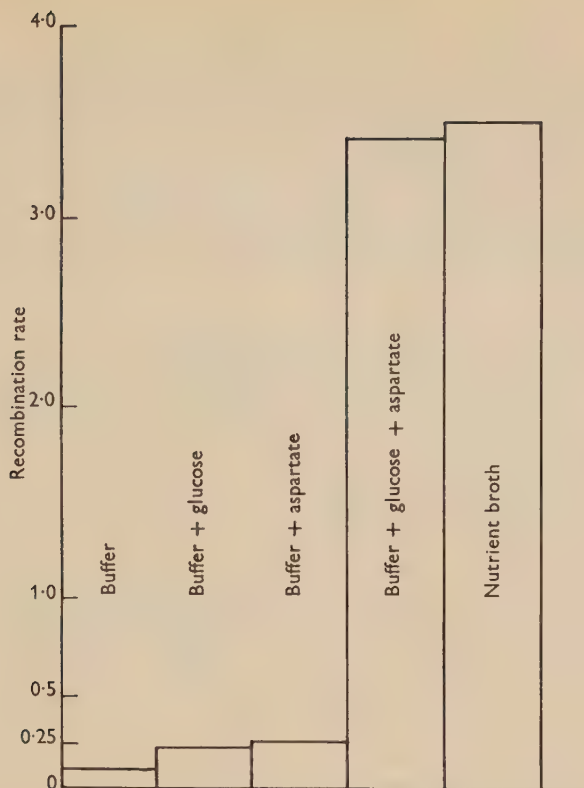


Fig. 1. Zygote formation in fluid media. Parent suspensions (58-161 Hfr and W-1 F-) were grown aerobically in broth, washed three times in buffer (pH 7.2), mixed and resuspended in the media shown. The buffer (pH 7.2) was supplemented as indicated with 20 μ mole glucose/ml. and 200 μ g sodium L-aspartate/ml. After incubating the mixture of parent cells for 30 min. the number of zygotes formed in each test medium was estimated.

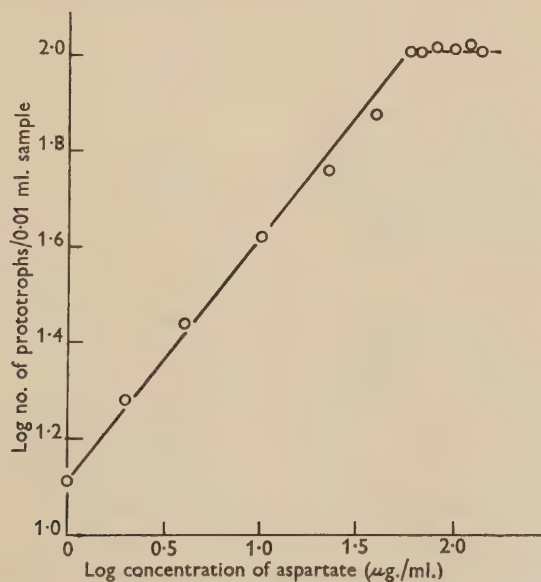


Fig. 2. The variation of recombination rate with the concentration of sodium L-aspartate. Parent cells (58-161 Hfr and W-1 F-) were washed with buffer (pH 7.2) and mated for 30 min. in buffer (pH 7.2)-glucose (20 μ mole/ml.)-aspartate. The number of zygotes formed at the end of this period was determined.

together, would enable NH_4^+ ions to be incorporated into the organic nitrogen of the cell. It was, however, ruled out by finding that nitrogen in the form of ammonium ions ($50 \mu\text{g. NH}_3\text{-N/ml.}$) did not stimulate zygote formation either in the presence or absence of glucose. Since the activity of L-aspartic acid can be reproduced by other substances having quite different stereochemical configurations, as the next section will show, it is unlikely that L-aspartic acid acts as an adsorption co-factor.

The effects on zygote formation in fluid media of replacing L-aspartic acid by other substances

A search was made for substances capable of replacing aspartic acid in promoting zygote formation in the presence of glucose. Most of the substances tested were substituted or unsubstituted dicarboxylic acids. They fell into three groups according to the effects they produced: (i) those having a stimulatory effect similar to that of L-aspartic acid; (ii) those without effect; (iii) substances inhibiting zygote formation.

The first group comprised DL-glutamic acid, succinic acid, fumaric acid and DL-malic acid. Of these, only DL-glutamic acid behaved in the same way as L-aspartic acid with regard to zygote formation. Succinic, fumaric and DL-malic acids did not raise the recombination rate when added to buffer +

Table 2. *The effect of pH value on the activity of succinic, fumaric and malic acids in zygote formation*

Parent suspensions were prepared in the standard manner (see Methods) and mated in the media show below. Substrate concentrations, unless specified otherwise, were the same in all experiments. Glucose: $20 \mu\text{mole/ml.}$; the acids (as sodium salts) were used at a concentration equivalent to $80 \mu\text{g. free acid/ml.}$

Parents mated in	Recombination rate at	
	pH 7.2	pH 6.5
Buffer	0.12	0.36
Buffer + glucose	0.36	1.0
Buffer + glucose + aspartate	2.8	7.1
Buffer + glucose + succinate	0.62	3.0
Buffer + glucose + fumarate	0.26	2.4
Buffer + glucose + malate	0.29	2.8

Viable counts of parent suspensions:

58-161 Hfr = 3.4×10^8 organisms/ml. W-1 F- = 2.9×10^8 organisms/ml.

glucose at pH 7.2 in the same molar concentrations as L-aspartic and DL-glutamic acids. However, when these substances were tested in buffer + glucose at pH 6.5 they showed considerable activity (Table 2) which was further accentuated at pH 5.8 (Table 6). From the results given in Table 6 it can be seen that in media buffered at pH 5.8 succinic, fumaric, DL-malic, L-aspartic and DL-glutamic acids stimulated the recombination rate to the same extent.

DL- α -Alanine, DL-tyrosine, DL-proline, DL-phenylalanine, oxalic, pimelic and adipic acids had no effect on the recombination rate. The fact that the latter substances were inactive suggested that the activity shown by the members

of the first group was not due solely to their dicarboxylic nature. In addition, the inactivity of the amino acids does not support the hypothesis that the importance of L-aspartic acid is due to the utilization of the α -amino nitrogen during the formation of a zygote.

Only malonic acid was observed to inhibit zygote formation. Cells of each parental strain (58-161 Hfr and W-1 F-) were grown and washed as previously described and then mixed in various media with and without malonic

Table 3. *The effect of malonate on zygote formation*

Suspensions of parent cells were prepared in the usual manner and mated in the media shown in the table which were buffered at pH 6.5. The concentration of sodium malonate used was equivalent to 6.5 mg. free acid/ml.

Parents mated in	Recombination rate	
	Without malonate	With malonate
Buffer	0.36	0
Buffer + glucose	1.0	0.32
Buffer + glucose + aspartate	7.1	3.0
Buffer + glucose + succinate	3.0	0.36
Buffer + glucose + fumarate	2.5	0.59
Buffer + glucose + malate	2.8	0.65

Viable counts on parent suspensions:

58-161 Hfr = 3.4×10^8 organisms/ml. W-1 F- = 2.9×10^8 organisms/ml.

acid. The results (Table 3) showed that malonic acid partially inhibited zygote formation. The recombination rate was reduced to a quarter or less of the rate observed in the absence of the inhibitor except in the case of aspartic acid.

It was found that acetate could replace glucose for the formation of zygotes when the mixture was buffered to pH 6.5. At higher pH values no evidence of replacement could be found, probably due to the non-penetration of the acetate ions. At pH 5.8 the parent cells were killed when suspended in buffer + acetate. It can be seen from Table 4 that the rate of recombination

Table 4. *A comparison of the effects of glucose and of acetate on zygote formation*

Parent suspensions were prepared in the usual way and mated in the media shown, buffered at pH 6.5. The concentration of sodium acetate was $40 \mu\text{mole/ml}$, other substrates were used at concentrations given in Table 2.

Parents mated in	Recombination rate	
	With glucose	With acetate
Buffer	0.21	1.6
Buffer + aspartate	6.9	3.7
Buffer + succinate	3.0	3.5
Buffer + fumarate	3.2	3.2
Buffer + malate	2.5	3.6

Viable counts on parental suspensions:

58-161 Hfr = 3.0×10^8 organisms/ml. W-1 F- = 2.9×10^8 organisms/ml.

in buffer + acetate alone was considerably higher than in buffer + glucose alone. This observation may possibly be due to the ability of *Escherichia coli* to couple two molecules of acetate to form succinate (Ajl & Kamen, 1950).

Zygote formation under anaerobic conditions

The tricarboxylic acid cycle is a catalytic pathway for the oxidation of 'C₂ fragments'. Its continued operation is dependent upon an adequate supply of oxygen which is utilized as the terminal hydrogen acceptor. If, as these experiments suggest, the formation of zygotes is dependent on the functioning of the Krebs cycle, then inhibition or stimulation of these reactions should also affect the recombination rate. The ability to form zygotes under anaerobic conditions was assessed as follows. The parent strains (58-161 Hfr V₆⁺ and W-1 F⁻ V₆⁺) were grown aerobically in nutrient broth, washed separately with three changes of buffer (pH 6.5) and resuspended in buffer at the same pH value. Samples of each parental suspension were transferred separately to different compartments of a series of Thunberg tubes each containing a different test medium (see Table 5). The tubes were flushed with nitrogen, evacuated and sealed. After warming to 37° the parental suspensions were mixed and rotated at 37° for 30 min. In a parallel aerobic experiment samples of the same parent suspensions and test media were used. The number of zygotes formed was estimated in the usual manner at the end of the 30 min. period. The experimental results given in Table 5 show clearly that zygotes were not formed at a significant rate under anaerobic conditions.

Table 5. *Comparison of the efficiency of zygote formation in various media under aerobic and anaerobic conditions*

Parent suspensions were grown and washed aerobically, and mixed under aerobic and anaerobic conditions in the test media shown, which were buffered at pH 6.5. The substrate concentrations shown in Table 2 were used.

Parents mated in	Recombination rate	
	Aerobic	Anaerobic
Buffer + glucose	0.25	0
Buffer + glucose + aspartate	5.7	0.04
Buffer + glucose + succinate	3.7	0.07
Buffer + glucose + fumarate	3.7	0.04
Buffer + glucose + malate	3.4	0.14
Buffer + glucose + glutamate	6.0	0.22

Viable counts on parental suspensions:

58-161 Hfr = 2.7×10^8 organisms/ml. W-1 F⁻ = 2.8×10^8 organisms/ml.

The effect of metabolic inhibitors on zygote formation

Fluoroacetic acid. Fluoroacetic acid acts as a poison by first being converted to fluorocitric acid which then competitively inhibits the metabolism of citric acid by the tricarboxylic acid cycle (Buffa & Peters, 1949; Liébecq & Peters, 1949; Lotspeich, Peters & Wilson, 1952). The ability of this inhibitor to stop zygote formation was accordingly investigated. As much as 75% inhibition

of zygote formation was achieved by using 10^{-3} M-sodium monofluoroacetate. The fluoroacetate did not inhibit zygote formation in buffer + glucose + dicarboxylic acid at pH values higher than pH 6. The concentration of glucose was important. The concentration normally used ($20\text{ }\mu\text{mole/ml.}$) had to be decreased to $2\text{ }\mu\text{mole/ml.}$ before definite inhibition of zygote formation was detected. This figure of $2\text{ }\mu\text{mole/ml.}$ is near the lowest concentration of glucose capable of supporting zygote formation and growth of the subsequent prototrophic clones on a minimal agar plate.

Table 6. *The effect of sodium monofluoroacetate on zygote formation*

Suspensions of parent cells were prepared by the standard procedure and mated under the conditions shown. The concentration of glucose in the media (buffered at pH 5.8) was decreased to $2\text{ }\mu\text{mole/ml.}$ Sodium monofluoroacetate was used at 10^{-3} M.

Parents mated in	Recombination rate	
	With fluoroacetate	Without fluoroacetate
Buffer	0.12	0.79
Buffer + glucose	0.31	1.0
Buffer + glucose + aspartate	2.9	6.4
Buffer + glucose + succinate	3.4	6.4
Buffer + glucose + fumarate	2.8	6.0
Buffer + glucose + malate	2.1	6.1
Buffer + glucose + glutamate	2.2	6.7

Viable counts on parent suspensions:

58-161 Hfr = 2.2×10^8 organisms/ml. W-1 F = 2.4×10^8 organisms/ml.

This effect of sodium monofluoroacetate affords further evidence that the tricarboxylic acid cycle is involved in zygote formation but it is not conclusive because of the incompleteness of the effect and also, as Elsdén (1954) has pointed out, because fluoroacetate may possibly inhibit any reaction in which a C_2 condensation with a C_x fragment is involved.

Sodium cyanide. Cyanide is known to inhibit the oxidation of reduced cytochrome 'c' by cytochrome oxidase. The oxidation of succinate to fumarate, and of malate to oxalacetate, both oxidative steps of the Krebs cycle, are linked to a cytochrome system either directly or through the intermediary of diphosphopyridine nucleotide (DPN). Table 7 shows that zygote formation between auxotrophic parent strains of *Escherichia coli* K-12 was almost completely inhibited in the presence of $M/500$ NaCN at pH 6.5. This result supports the hypothesis that the efficient operation of the Krebs cycle is necessary for the formation of zygotes, although it must still be borne in mind that all other reactions dependent on terminal oxidations by means of cytochrome systems will also be affected by this particular inhibitor.

Dinitrophenol. The functions of the Krebs cycle are twofold. One is to provide the cell with an adequate supply of intermediates which can be further modified to provide amino acids (Roberts *et al.* 1953; Abelson *et al.* 1953). The other is the release of energy, associated with hydrogen transport by the cellular cytochromes and its final oxidation by molecular oxygen. This

Table 7. *The effect of sodium cyanide on zygote formation*

Washed parent suspensions were mated in the media shown (buffered at pH 6.5), the substrate concentrations used were the same as shown in Table 2. The concentration of sodium cyanide used was $2 \times 10^{-3} \text{ M}$.

Parents mated in	Recombination rate	
	With cyanide	Without cyanide
Buffer	0	0.5
Buffer + glucose	—	1.5
Buffer + glucose + aspartate	0.2	7.2
Buffer + glucose + succinate	0.5	3.2
Buffer + glucose + fumarate	0.2	3.0
Buffer + glucose + malate	0.3	3.5

Viable counts on parental suspensions:

58-161 Hfr = 3.3×10^8 organisms/ml. W-1 F- = 2.9×10^8 organisms/ml.

free energy is made available to cells by the formation of high energy bonds associated with adenosine triphosphate (ATP). ATP is able to donate its terminal phosphate group together with the free energy associated with it to certain classes of substances, forming phosphorylated derivatives. Such phosphorylated substances, having a higher free energy content, can take part in reactions which, on thermodynamic grounds, would be denied to the corresponding non-phosphorylated substance. In this way respiration is coupled with synthesis. It has been demonstrated that certain substances can prevent the accumulation of ATP. Among these are gramicidin, atebirin and 2:4-dinitrophenol. The work of Loomis & Lipmann (1948) and especially Teply (1949) has shown that 2:4-dinitrophenol acts as an uncoupling agent (i.e. disconnecting synthesis from respiration) by degrading ATP to inorganic phosphate. Table 8 shows the results of crosses between 58-161 Hfr and W-1

Table 8. *The effect of 2:4-dinitrophenol (DNP) on zygote formation*

The cell suspensions used in the particular experiment reported here were the same as those used for the experiment reported in Table 7. Substrate concentrations employed were also the same. Final concentration of 2:4-dinitrophenol was 10^{-3} M .

Parents mated in	Recombination rate	
	With DNP	Without DNP
Buffer	0	0.5
Buffer + glucose	0.07	1.5
Buffer + glucose + aspartate	0.31	7.2
Buffer + glucose + succinate	0.14	3.2
Buffer + glucose + fumarate	0.17	3.0
Buffer + glucose + malate	0.17	3.5

Viable counts on parental suspensions:

58-161 Hfr = 3.3×10^8 organisms/ml. W-1 F- = 2.9×10^8 organisms/ml.

F- in the presence of 10^{-3} M 2:4-dinitrophenol. It can be seen that this substance prevented zygote formation. Thus one may conclude that the process of zygote formation is endergonic.

In connexion with the use of inhibitors it was established that 10^{-3}M -2:4-dinitrophenol, $2 \times 10^{-3}\text{M}$ -NaCN and 10^{-3}M -sodium monofluoroacetate had no detectable effect on the viability of the parental suspensions throughout the experimental period.

Chloramphenicol. Wisseman *et al.*' (1954) demonstrated the specific inhibition of protein synthesis in *Escherichia coli* by growth inhibitory levels of chloramphenicol. The inhibition was complete within 10 min. of addition of the antibiotic to the cells. Table 9 shows the effect of chloramphenicol on zygote formation; the recombination rate was unaltered by $10\text{ }\mu\text{g.}$ chloramphenicol/ml. Growth of the donor strain was prevented by $5\text{ }\mu\text{g.}$ chloramphenicol/ml. These results suggest that protein synthesis is not specifically associated with zygote formation.

Table 9. *The effect of growth inhibitory concentrations of chloramphenicol and 8-azaguanine on zygote formation in Escherichia coli*

Washed parent suspensions of strains 58-161 Hfr and W-1 F- were mated under the conditions shown. The media, buffered at pH 6.5, contained the same substrate concentrations as shown in Table 2. The concentration of chloramphenicol used was $10\text{ }\mu\text{g./ml.}$ and, where indicated, $200\text{ }\mu\text{g.}$ 8-azaguanine/ml. The number of zygotes formed was estimated after 30 min. incubation at 37° in the usual manner.

Parents mated in	Recombination rate
Buffer + glucose + aspartate	6.3
Buffer + glucose + aspartate + 8-azaguanine	6.5
Buffer + glucose + aspartate + chloramphenicol	6.3
Nutrient broth	7.5
Nutrient broth + 8-azaguanine	7.0
Nutrient broth + chloramphenicol	7.1

Viable counts on parental suspensions:

58-161 Hfr = 2.0×10^8 organisms/ml. W-1 F- = 1.6×10^8 organisms/ml.

8-Azaguanine. Inhibition of nucleic acid synthesis has been achieved using a variety of inhibitors, among them ultraviolet light (Kelner, 1953), nitrogen mustard and 8-azaguanine (Skipper *et al.* 1951). 8-Azaguanine is non-specific in that it inhibits the synthesis of both pentose and deoxypentose nucleic acids. 8-Azaguanine at $200\text{ }\mu\text{g./ml.}$ inhibited growth of the donor parent (58-161 Hfr) in fluid minimal medium supplemented with glucose and methionine; this concentration, however, did not affect the recombination rate either in buffer + glucose + aspartate (pH 6.5) or in nutrient broth (Table 9).

It therefore seems unlikely that synthesis of either kind of nucleic acid is essential to zygote formation.

Requirements for zygote formation between 58-161 F+ and W-1 F-

Some of the experiments reported in the previous sections were repeated with the strains 58-161 F+ and W-1 F-. Experiments with an F+ \times F- system demand some small alterations of technique. Since the recombination rate is some 1000 times lower than in Hfr \times F- crosses the zygote suspension must be plated relatively undiluted, so that the nutrient broth in which

phage T_6 is suspended, together with the unabsorbed phage, must be removed by washing the cells with two changes of buffer after treatment with coliphage. The results observed (Table 10) paralleled those found with the equivalent $Hfr \times F^-$ cross, i.e. an energy source (glucose) was required together with aspartic acid, which could be replaced with succinic acid, fumaric acid or malic acid. 2,4-Dinitrophenol inhibited zygote formation.

Table 10. *Zygote formation by strains 58-161 F^+ V_6^+ and W-1 F^- V_6^-*

Parental cell suspensions were prepared by washing aerobically grown cells with buffer (pH 7.2). The cells were mixed in the test media shown (pH 6.2) and incubated aerobically for 30 min. at 37°. The number of zygotes formed was estimated by treatment of the sample with phage T_6 , washing the cells twice with buffer and resuspending to the original volume before plating on minimal agar + thiamine + glucose.

Parents mated	No. of T + L + recombinants per 0.1 ml. plated
Buffer	20
Buffer + glucose	49
Buffer + glucose + aspartate	334
Buffer + glucose + succinate	160
Buffer + glucose + fumarate	148
Buffer + glucose + malate	179
Buffer + glucose + 10^{-3} M-DNP*	5
Buffer + glucose + aspartate + 10^{-3} M-DNP	10

* DNP = 2,4-dinitrophenol.

DISCUSSION

It will be convenient to repeat the three suggestions made previously to account for the action of L-aspartic acid during zygote formation. These were: (i) its possible importance as an essential intermediate of a biosynthetic pathway; (ii) its action as an adsorption co-factor necessary for the effective union of donor and recipient cells; (iii) its functioning as a means of entry to the Krebs cycle for C_4 fragments capable of increasing the rate of oxidation of C_2 fragments derived from glucose.

Of the amino acids tested for their effect on zygote formation only glutamic acid showed the same effect as aspartic acid. Ammonium ion had no effect on the recombination rate under any conditions tested, a fact which makes it extremely unlikely that aspartate influences recombination through transamination reactions. Moreover, substances not containing α -amino-nitrogen can stimulate the recombination rate. Furthermore, it was shown that chloramphenicol, which is known specifically to inhibit protein synthesis in *Escherichia coli* (Wisseman *et al.* 1954), does not prevent zygote formation when used at concentrations which rapidly prevent cell proliferation. Protein synthesis seems therefore not essential for zygote formation.

Reichard (1954) showed that aspartic acid is a precursor of ureidosuccinic acid and ultimately of orotic acid which possesses the basic pyrimidine structure. Aspartic acid might therefore act as a precursor of pyrimidines in

nucleic acid synthesis and thus be required for zygote formation. Swenson (1950) showed that inhibition of the adaptive synthesis of galactozymase in yeasts by ultraviolet light (u.v.) was probably due to a primary effect on nucleic acid synthesis; Pollock (1953) reached a similar conclusion. Kelner (1953) subsequently showed that u.v.-irradiation specifically and rapidly inhibited deoxyribonucleic acid synthesis in *Escherichia coli*, yet treatment of donor strains of *E. coli* K-12 with small doses of u.v. light reduces their fertility little or not at all when the strains are mated immediately after u.v.-irradiation (Hayes, 1953*b*). It seems, therefore, unlikely that deoxyribonucleic acid synthesis is an essential prerequisite of zygote formation in *E. coli* K-12. This conclusion is confirmed by the observation that 8-azaguanine had no effect on the recombination rate when used at concentrations which were inhibitory to growth.

It is difficult to accept suggestion (ii) above in view of the evidence presented in Fig. 1, which shows that aspartic acid alone did not stimulate the recombination rate. Moreover, substances other than aspartic acid can show the same activity in zygote formation. However, these substances might lead to the endogenous production of aspartic acid which might then be excreted.

Saz & Krampitz (1954) concluded that the tricarboxylic acid cycle was fully functional in *Escherichia coli*, from their analysis of the distribution of ^{14}C derived from acetate-2- ^{14}C among isolated intermediates of the tricarboxylic acid cycle. Repaske & Wilson (1953), with extracts of *Azotobacter agile*, demonstrated the 'sparking effect'. This phenomenon is essentially the catalytic oxidation of acetate by a C_4 dicarboxylic acid intermediate of the tricarboxylic acid cycle. It seems likely that this is the explanation of the results of experiments such as shown in Fig. 1 where it can be seen that the combined effect of glucose and aspartic acid on the recombination rate greatly exceeded the sum of their individual effects.

Johnson & Cohn (1952) showed that the stimulation of total growth of *Escherichia coli* produced by the addition of fumarate or malate to a complete culture medium could not be produced by the addition of an equivalent amount of glucose. They suggested that the stimulation was due to increased amounts of free energy made available to the cells. Among the substances tested for their ability to stimulate the recombination rate only those known intermediates of the Krebs cycle, i.e. succinic, fumaric and malic acids or their immediate precursors, e.g. glutamic acid, were able to do so. At low pH values the activity of all the dicarboxylic acids in stimulating the recombination rate was identical. This effect of the pH value (Tables 2 and 6) may reflect the greater ease of penetration of the non-amino dicarboxylic acids into the cells at lower pH values (Moses, 1955). The results obtained by using metabolic inhibitors support to varying degrees the suggestion that it is the supply of energy made available by the oxidation of acetate via the tricarboxylic acid cycle which is essential for zygote formation.

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The Nature of the Endergonic Processes in Conjugation in *Escherichia coli* K-12

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SUMMARY: The energy required during zygote formation was found to be needed solely by the donor parent. The free energy is utilized by the donor cell in the establishment of effective contacts and subsequently in the transfer of the genetic material to the F⁻ cell. Energy is not required to hold the cells in contact throughout the mating process. The effect of pH value on the recombination rate is described.

Zygote formation in *Escherichia coli* K-12 is an endergonic process which is probably not dependent on protein or nucleic acid synthesis (Fisher, 1957). This paper describes an analysis of zygote formation in order to establish more precisely the nature of the endergonic stages.

METHODS

The materials and bacterial strains used have been described previously (Fisher, 1957). In the present experiments, however, beef-digest broth was replaced by casein hydrolysate yeast extract broth (CHYE) (Difco 'Casamino acids', Technical, 2% (w/v) + Difco dehydrated yeast extract, 0.5% (w/v); pH value adjusted to 7.4). *Escherichia coli* K-12, strains 58-161 Hfr (Hayes, 1953) and W-1 F⁻ (Lederberg & Lederberg, 1952) were used exclusively.

The methods used to grow the parent cultures and to detect the formation of zygotes were as described by Fisher (1957). Other experimental procedures varied with the demands of particular experiments and will be described in their proper place in the text.

RESULTS

It was shown (Fisher, 1957) that free energy is required for zygote formation. An analysis of this energy requirement must first resolve whether the energy is required by one or both parents and, secondly, the particular stage or stages of conjugation which are endergonic.

The energy requirements of each parent during conjugation

Though optimal zygote formation occurs in buffer + glucose + aspartate, zygotes are still formed at a low rate in unsupplemented buffer. It was thought that the low rate which occurred in buffer might be due to endogenous carbohydrate reserves of the bacteria. The effect of starvation on washed parent cells was therefore investigated. The cells were differentially depleted of their carbohydrate or nitrogenous reserves by aeration at 37° in buffer supplemented

with ammonium chloride or with glucose, respectively. Depletion of nitrogenous material of either or both parents did not decrease the recombination rate. The effect of carbohydrate depletion is shown in Table 1. It is clear that carbohydrate depletion of the Hfr parent alone affected the rate of zygote

Table 1. *The effect of starvation of parent organisms on zygote formation*

Parent cells were grown aerobically and starved by aerating in buffer (pH 7.2) at 37° for 2 hr. Starved and unstarved cells were mated for 30 min. at 37° in 0.05 M-phosphate buffer (pH 7.2) and then the mixtures assayed for the number of T+L+ recombinants by diluting the zygote suspension (2×10^{-4}) and plating triplicate samples on minimal agar supplemented with thiamine and glucose.

Cross	No. of T+L+ recombinants/0.01 ml. sample
Hfr starved \times F- starved	6
Hfr starved \times F- unstarved	7
Hfr unstarved \times F- starved	110
Hfr unstarved \times F- unstarved	114

formation; the recipient F- parent displayed no requirement for energy during conjugation. Such a distinction between the parent strains is of interest since it parallels the differentiation by Hayes (1952) of donor and recipient strains, recently confirmed by the elegant experiments of Wollman & Jacob (1955) on the inheritance of lambda prophage, and the direct observations of Lederberg (1956).

The energy-requiring stages of zygote formation

Zygote formation in *Escherichia coli* has been subdivided into a stage of effective contact between donor (Hfr) and recipient (F-) cells, followed by the transfer of genetic material (Wollman & Jacob, 1955; Jacob & Wollman, 1955). These workers showed that genetic transfer is effected by the penetration of the F- cell by a specific extremity of the Hfr chromosome which is then followed by other genetic loci in the same sequence as their arrangement along the chromosome. When mating cells are separated at intervals of time by a Waring Blendor it is found that the Hfr selective markers which control threonine and leucine independence (T+L+) enter the F- cells (T-L-) after a lag of *c.* 8 min. after mixing the parent cells. When mating cells are separated at this time it is found that prototrophic recombinants (T+L+) have inherited few or none of the unselected markers of the Hfr parent. As with T+L+, there is a characteristic time at which the unselected loci first begin to be detectable among T+L+ recombinants. Thus, about 9 min. after mixing, the Hfr locus for resistance to sodium azide (Az^r) begins to appear; resistance to phage T₁ (T₁^r) appears at 10 min.; ability to ferment lactose (Lac₁+) and galactose (Gal_b+) appears at 18 and 28 min. respectively. In the Hfr \times F- cross only part of the Hfr chromosome is transferred and this is completed in about 30 min. These findings have been confirmed by using virulent phage specifically to kill the Hfr parent instead of separating the mating pairs by agitation (Hayes, 1957).

Investigation of the lag period

In a medium which supports zygote formation use of the Waring Blendor or of virulent phage can only give information about zygote formation subsequent to the time at which the first and succeeding Hfr loci enter the F-cell and allow the formation of detectable recombinants. An attempt was made to investigate the earlier stages of zygote formation with the specific

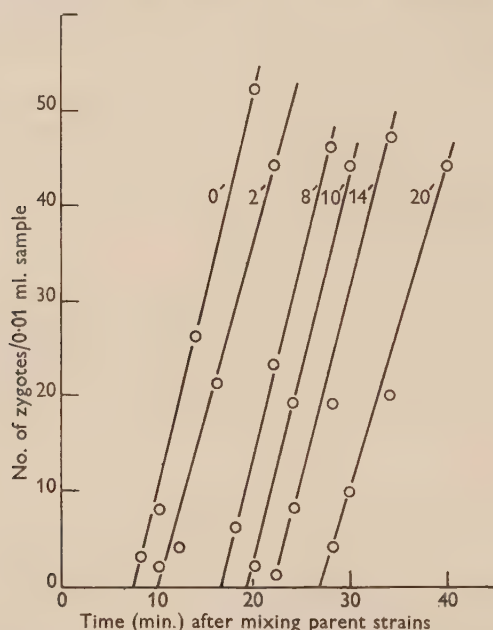


Fig. 1. The effect of an initial anaerobic period on zygote formation. Washed suspensions of parent strains (58-161 Hfr V_6^- and W-1 F $-$) were pipetted into separate sections of a series of Thunberg tubes each of which contained buffer (pH 6.5)+glucose (20 μ mole/ml.)+sodium aspartate (equivalent to 80 μ g./ml.) free acid. After flushing with nitrogen the tubes were evacuated, sealed and warmed to 37° before mixing the contents. Each tube in turn was oxygenated and thereafter sampled at intervals for the number of T+L+ recombinants. The figures on the curves indicate the time after mixing at which oxygen was admitted to the mixtures.

aim of detecting stages which might occur in the absence of available free energy. Since oxygen is required for zygote formation (Fisher, 1957), the simplest approach to this problem was to see whether any step preceding detectable genetic transfer would occur under anaerobic conditions. A series of identical suspensions of Hfr and F $-$ cells in buffer+glucose+aspartate were mixed and shaken gently in Thunberg tubes at 37° under anaerobic conditions (Fisher, 1957). The tubes were oxygenated in turn at intervals after mixing. Thereafter samples were removed at intervals and assayed for the number of zygotes formed as shown by the number of T+L+ recombinants present by using a modification of the phage method (see below); the results are shown in Fig. 1. It can be seen that all the curves which describe the rate of zygote formation are parallel, but each is displaced from the control

curve (oxygenated at zero time) by an interval of the same duration as the anaerobic period which preceded oxygenation. This shows that under anaerobic conditions nothing happened which affected the efficiency of subsequent conjugations, although the probability of collision was the same as in the presence of oxygen. From this it is concluded that zygote formation is an energy-requiring process from its inception, so that the formation of effective contacts is endergonic.

Effect of pH value on recombination

Arising from observations associated with the penetration of dicarboxylic acids into parent cells (Fisher, 1957), the effect of the pH value of the environment on the recombination rate was investigated. To avoid complications due to ionizable substrates (dicarboxylic acids) the determinations were carried out in unsupplemented buffer where the mating processes are forced to rely

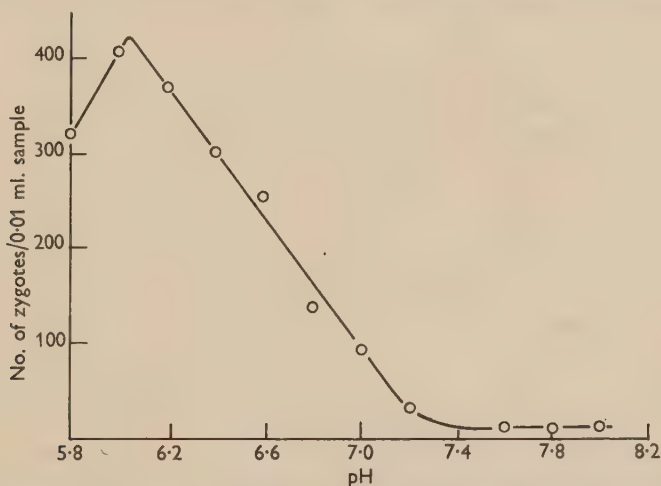


Fig. 2. The effect of pH value of medium on zygote formation between strains 58-161 Hfr and W-1 F-. Parent organisms were washed separately with 0.05 M-phosphate buffer (pH 7.2) and then resuspended and mixed in 0.05 M-phosphate buffer containing 0.4 % (w/v) NaCl and 0.02 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH values of the buffers were accurately adjusted (Muirhead pH meter). The mixtures were assayed for the number of zygotes formed after 30 min. incubation at 37°.

on the endogenous reserves of the Hfr cells. Hfr and F- cell suspensions were mixed in 0.05 M-phosphate buffer accurately adjusted to different pH values measured electrometrically. The mixed suspensions were aerated at 37° for 30 min. and each assayed for the number of zygotes formed; the results are shown in Fig. 2. There were marked differences in the recombination rate at different pH values. The optimal pH value for the strains used was 6.1. Since the effect of pH value in these experiments was unrelated to energy supply, it is most probably ascribable to alterations in the distribution of ionized groups on the surface of the cells. Maccacaro (1955) and Maccacaro & Comolli (1956) reached the conclusion that surface groupings are intimately concerned with the fertility of F+ × F- crosses.

Chromosome transfer: an endergonic process

It has been shown above that energy is required for the formation of effective contacts. The question remains, does the energy requirement extend throughout the whole period of zygote formation, i.e. is energy needed by the Hfr cell for chromosome transfer? The action of a number of inhibitors on zygote formation has been described (Fisher, 1957). Of these substances, 2:4-dinitrophenol (DNP) was chosen for the purpose of determining whether chromosome transfer could be inhibited by the uncoupling of oxidative phosphorylation and free energy utilization. Since the action of DNP is reversible it was found necessary to kill the Hfr cells with phage T_6 (Hayes, 1957), to which the F-cells are resistant, before diluting out the DNP as a preliminary to plating on selective medium for recombinants. Washed parent suspensions were mixed in buffer + glucose + aspartate (pH 6.5) and aerated at 37° . At intervals thereafter samples were removed to DNP (final conc. 10^{-3}M) and aerated at 37° . The samples containing DNP were then scored as a function of time for T + L + and T + L + Lac + recombinants by treatment with phage T_6 followed by dilution and plating on minimal agar + thiamine + glucose (MAB₁: selection for T + L + only) and on minimal agar + thiamine + lactose (MAB₁Lac: selection for T + L + Lac +). The results are given in Fig. 3. Curve 1 shows the total number of T + L + recombinants found at various times after mixing the parents; curve 5 shows the number of T + L + Lac + recombinants found in the same samples. The horizontal lines represent the number of recombinants of either class arising from samples added to DNP at 10, 15 and 20 min. after mixing. If the effect of adding DNP was to interfere only with effective contact formation and not with transfer, one would expect to find, in any sample, a proportion of pairs of cells which had already made effective contact but in which transfer of T + L + had not occurred. If, therefore, DNP did not interfere with chromosomal transfer, the proportion of T + L + recombinants should rise after addition of DNP. Furthermore, in the case of those zygotes which had acquired T + L + (e.g. curve 2) but not Lac + at the time of adding DNP, the proportion of T + L + Lac + recombinants (curve 6) should increase. The fact that the curves are horizontal shows that chromosome transfer is inhibited by DNP.

The use of 2:4-dinitrophenol in the analysis of chromosome transfer

The kinetics of chromosome transfer has been determined by using DNP in combination with phage T_6 . Washed parent suspensions were mixed in buffer + glucose + aspartate (pH 6.5) and, at intervals after mixing, samples were removed to DNP (final conc. 10^{-3}M) and, after a convenient interval, were treated with phage T_6 . The samples were then diluted and plated on minimal agar + glucose + thiamine + L-leucine (MAB₁L: selection for T + transfer only). Control plates of MAB₁L spread with double-sized samples of either parent alone showed no growth after incubation. Recombinant clones arising from each sample of the mixture were purified and scored for inheritance from the Hfr parent of the unselected markers: L + Az^rV₁Lac + V₆^s. The

results of these analyses are plotted in Fig. 4. From the time at which it was first detectable, the quantitative increase with time of any particular Hfr gene among the T⁺ recombinants could be demonstrated. The order of transfer of genes was found to be the same as the order of their arrangement on the chromosome as established by other authors from recombination data (Lederberg, 1947; Cavalli-Sforza & Jinks, 1956). With the method described here it was possible to separate in time the transfer of the closely linked loci T⁺, L⁺ and Az^r.

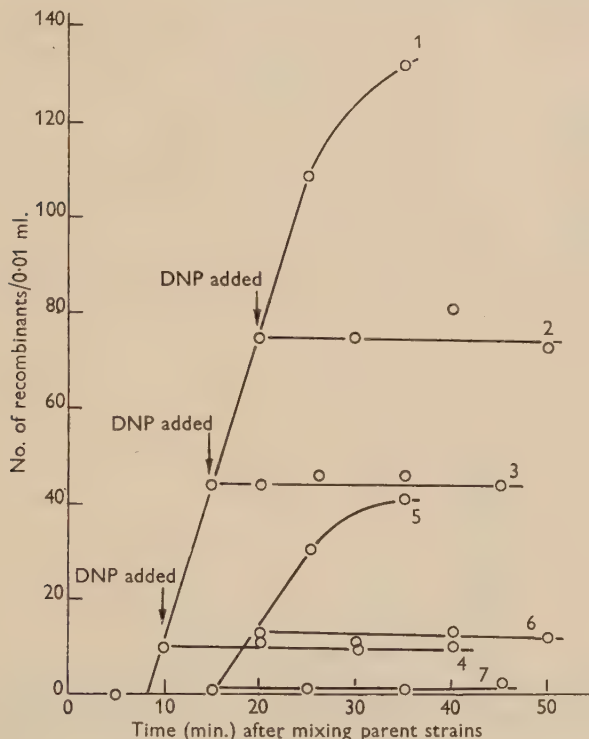


Fig. 3. The effect of 2:4-dinitrophenol on chromosome movement. Suspensions of washed parent cells (58-161 Hfr V_8^+ and W-1 F⁻ V_8^+) were mixed in buffer + glucose + aspartate. At intervals after mixing samples were removed to DNP solution (10^{-3} M) and following treatment with phage T₆ were diluted (2×10^{-4}) and 0.01 ml. samples plated on minimal agar + thiamine + glucose (MAB₁: selection for T⁺ + L⁺ inheritance from the Hfr parent, curve 1) and on minimal agar + thiamine + lactose (MAB₁Lac: selection for inheritance of T⁺ + L⁺ and Lac⁺ from the Hfr parent, curve 5). At 10, 15 and 20 min. after mixing, larger samples were removed to DNP (10^{-3} M) and thereafter subsamples were treated with phage T₆, diluted and plated on MAB₁ (curves 2-4) and on MAB₁Lac (curves 6 and 7).

The continuity of chromosomal transfer

Experiments were designed to test the assumption made in this paper and that of Wollman & Jacob (1955) that the kinetic results obtained reflect the movement of a physical continuum from the donor to the recipient cell. Parent suspensions were mixed in buffer + glucose + aspartate (pH 6.5) and

aerated at 37° for 20 min. From previous experiments it was known that at this time the Hfr loci T+L+ had been transferred to the majority of zygotes but that the locus Lac+ was only beginning to enter these zygotes. At this point the mixture was cooled to 4° and diluted 1/100 with buffer+glucose+aspartate at 4° to prevent further collisions. After 20 min. at 4° the mixture was rapidly warmed to 37° and aeration continued. At intervals throughout

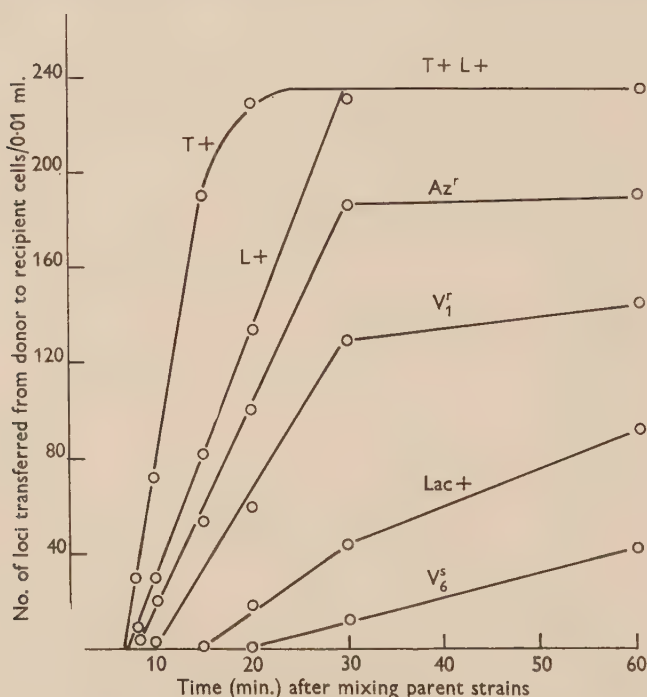


Fig. 4. The variation of recombinant genotype with the period of mating between 58-161 Hfr and W-1 F-. Washed suspensions of parent cells (58-161 Hfr T+L+B₁+M-, Az^r, V₁^r, Lac₁+, V₆^s and W-1 F- T-L-B₁-M+ Az₁^sV₁^sLac₁-V₆^s were mixed in buffer+glucose+aspartate (pH 6.5) (see Fig. 1 for substrate concentrations). At intervals after mixing samples were removed to DNP (10⁻³M)+phage T₆ and incubated for 10 min. After dilution triplicate 0.01 ml. samples were plated on minimal agar+thiamine+glucose+leucine (selection for T+inheritance from Hfr cell). Recombinant clones were purified and examined for the inheritance of unselected markers from the Hfr parent.

the entire period samples were transferred to DNP+phage T₆ and finally plated to assess the number of T+L+ and of T+L+Lac+ recombinants. The results are given in Fig. 5, where it can be seen that increase in all classes of recombinants was completely prevented by cooling. After rewarming the entry of Lac+ was resumed at its initial rate. Since, over the same period of time, the number of T+L+ recombinants increased only slightly, the increase of T+L+Lac+ recombinants cannot be explained on the basis of formation of new recombinant pairs. It must, therefore, be due to the subsequent entry of Lac+ into zygotes which, at the time of cooling, had

already acquired T + L+ but had not separated during the period at 4°. This experiment confirms the results obtained with DNP and rules out the possibility that energy is also required to hold the cells in contact throughout transfer.

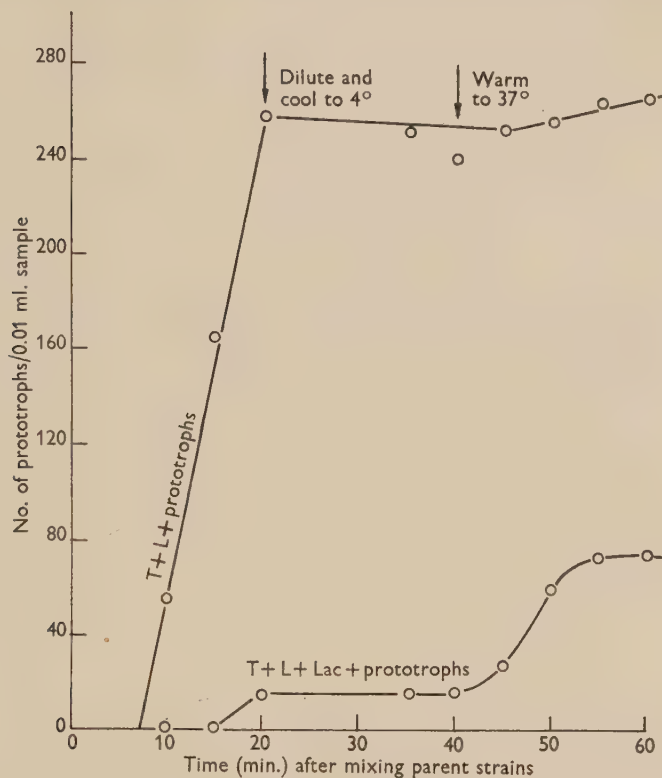


Fig. 5. The continuity of chromosomal transfer. Washed suspensions of 58-161 Hfr (M- Lac + V_6^+) and W-1 F- (TLB₁- Lac- V_6^+) were mixed in buffer + glucose + aspartate (pH 6.5) and aerated at 37° for 20 min. The mixture was then cooled to 4° and diluted 1/100 in buffer + glucose + aspartate at 4°. After 20 min the mixture was rapidly warmed to 37° and aeration at 37° continued. Samples were removed at intervals to DNP + phage T₆ and then diluted (2×10^{-4}) and 0.01 ml. samples plated in triplicate on: (1) minimal agar + thiamine + glucose (selection for inheritance of T + L+ only from the Hfr parent); (2) minimal agar + thiamine + lactose (selection for inheritance of T + L+ and Lac+ from the Hfr parent).

DISCUSSION

It has been shown that energy is required for zygote formation (Fisher, 1957). This energy is utilized only by the donor cells in both Hfr and F+ crosses with F- cells. Thus the F- cells play a passive role during this stage of the mating process, although, later, they must be responsible for supporting the stages of recombination and segregation. Zygote formation comprises everything that happens from the initial collision between donor and recipient cells until the completion of chromosome transfer. The ability of donor and recipient cells to form an intimate contact following collision is probably

decided by their surface configurations (Maccacaro, 1955; Maccacaro & Comolli, 1956). The effect of pH value of the suspending fluid in altering the recombination rate thus becomes understandable if it exercises a surface effect which may alter the mutual attraction between the cells. It has been shown (Hayes, 1957) that alteration in pH value has the same effect on the mating process as variation in population density, which only affects the rate of collision and is not related to energy-requiring processes. The effect of the pH value of the medium is, therefore, probably to increase the proportion of collisions which are followed by adherence.

However, in the absence of a supply of free energy no spontaneously irreversible step in conjugation takes place, i.e. energy is required for the formation of effective contacts. The fact that energy is also required for chromosome transfer raises the question whether the irreversible link uniting the cells is really the chromosome itself. Electron microphotographs show the presence of a bridge of material between conjugating cells (Wollman, Jacob & Hayes, 1956). This bridge is clearly not the chromosome, as the absence of effect of DNA-ase on recombination had already suggested (Lederberg, 1947; Hayes, 1957). The appearance of some of these bridges implies that they must exercise some uniting force between the pairs of cells, and it might be suggested that the energy required for effective contact formation is utilized in making them. Against this is the finding that chloramphenicol, which inhibits protein synthesis in *Escherichia coli* (Wisseemann, Smadel, Hahn & Hopps, 1954), does not appear to inhibit zygote formation (Fisher, 1957). These experiments may, however, be criticized, since the chloramphenicol was only added to the mating cells at the time of mixing. Curves expressing the rate at which effective contacts are formed arise from the origin (Wollman & Jacob, 1955) so that this essential step of the mating process must be accomplished very rapidly. It is therefore possible that effective contacts had been formed before protein synthesis was arrested. It seems clear, however, that protein synthesis is not essential to chromosome transfer.

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The Pathway of Breakdown of 2:4-Dichloro- and 4-Chloro-2-methyl-phenoxyacetic Acid by Bacteria

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SUMMARY: The metabolism of 2:4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid by a strain of *Flavobacterium peregrinum* and an *Achromobacter* sp., respectively, has been studied. Bacteria from young cultures were more active than those from older ones in oxidizing these substrates. Evidence is presented that adapted organisms dissimilate 2:4-dichlorophenoxyacetic acid through 2:4-dichlorophenol and 4-chlorocatechol, and that 4-chloro-2-methylphenoxyacetic acid is dissimilated through 5-chloro-2-cresol. Bacteria grown on 2:4-dichlorophenoxyacetic acid do not oxidize any of the other five possible isomers, but can oxidize 2:4-dibromo-, 4-bromo-2-chloro-, 4-chloro-, and, to a small extent, 2-chlorophenoxyacetic acids.

That the selective herbicides, 2:4-dichlorophenoxyacetic acid (2:4-D) and 4-chloro-2-methyl-phenoxyacetic acids (MCPA) are subject to bacterial decomposition in the soil is now well known, and the fate of these substances in bacterial cultures has been investigated by a number of workers. Evans & Smith (1954) suggested that 6-hydroxy-2:4-dichlorophenoxyacetic acid was an early oxidation product of 2:4-D in a culture of an unidentified bacterium. Audus (1952) used a soil-perfusion technique to study the kinetics of the decomposition of herbicides in fresh soil and obtained some evidence that 2:4-dichlorophenol was an intermediate in 2:4-D breakdown. He found that a soil enriched with organisms to decompose 2:4-D would also decompose MCPA and conversely, and so argued that the first step in decomposition was an attack on the part of the molecule common to both compounds, namely, the acetic acid residue, which he postulated was split off by hydrolysis to glycollic acid, leaving the corresponding phenol. However, in more recent work with liquid cultures of *Bacterium globiforme* Audus & Symonds (1955) claimed to have detected at least two phytotoxic intermediates, differing from 2:4-D in R_f values by paper chromatography, and so they suggested that the implications of their earlier cross-perfusion experiments would have to be reconsidered. Rogoff & Reid (1956) isolated from soil a yellow *Corynebacterium* sp. which could decompose up to 0.3% 2:4-D in a mineral salts medium. This organism oxidized 3.8, 19 and 38 μM 2:4-D in Warburg manometers, but 38 μM showed a toxic effect; the organism also oxidized 2:4-dichlorophenol. They detected in cultures the nearly quantitative conversion of the organically-bound chlorine in 2:4-D to the inorganic form and so concluded that the molecule suffered complete decomposition. Steenson & Walker (1956), using cultures of two different herbicide-decomposing bacteria, showed that the bacterial oxidation of 2:4-D or MCPA depended on adaptive (induced) enzyme

formation. No oxidation of either 6-hydroxy-2:4-dichlorophenoxyacetic acid or of 2:4-dichlorophenol at the concentration used was obtained, and it was concluded that neither compound was an intermediate in 2:4-D breakdown.

In the present work, the principle of simultaneous adaptation (Stanier, 1947) has been employed to study the metabolism of 2:4-D, MCPA and some related compounds by the two bacterial species which were described by Steenson & Walker (1956). Organisms for manometric experiments were grown on mineral salts agar plates with 2:4-D or MCPA as carbon source and supplemented with a little yeast extract. Growth, however, was slow and it was necessary to incubate for 5, 6 or more days to obtain a moderate amount of organisms. When cultures were grown for not more than 3 days, even though the yield of organisms appeared small, these organisms were found to be much more active than older ones; this observation was the key to many of the results which follow.

METHODS

Medium. Agar plates were poured from a medium containing 2:4-D, 0.1 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.05 g.; KCl, 0.02 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g.; Difco dehydrated yeast extract, 0.05 g.; agar, 2.0 g. in tap water, 100 ml. When necessary, MCPA was used in place of 2:4-D. All cultures were incubated at 25°.

Manometric methods. Bacteria were washed in 0.02 M-phosphate buffer (pH 6.98) and resuspended in the same buffer solution. The nitrogen content of the suspension was determined by the usual micro-Kjeldahl procedure. The oxygen uptake by 1 ml. cell suspension (containing 0.2–0.6 mg. total N) was measured in Warburg manometers at 30°. The main cup of the Warburg vessel contained the suspension of organisms and enough phosphate buffer to give a final volume of 3 ml., the substrates being placed in the side bulb. The centre cup contained 0.2 ml. 20% (w/v) aqueous potassium hydroxide.

Analytical methods. Chloride was determined by Mohr's method using 0.05 N-silver nitrate solution (Kolthoff & Sandell, 1952). Phytotoxic effects (Audus & Quastel, 1947) were detected by placing a 2–3 ml. sample in a Petri dish together with a piece of filter-paper and planting twenty mustard or cress seeds on the paper. The seeds used had a percentage germination of over 90%.

Reference compounds. 3-Chloro-, 3:4-dichloro and 2:4-dibromo-phenoxyacetic acids were synthesized from the corresponding phenols by the method of Pokorny (1941). 2:3-, 2:5- and 3:5-dichlorophenoxyacetic acids were given to us by Professor R. L. Wain; 2:5- and 2:6-dichlorophenoxyacetic acids by Professor G. E. Blackman; 6-hydroxy-2:4-dichlorophenoxyacetic acid by Dr G. W. K. Cavill; 2-hydroxy-4-chlorophenoxyacetic acid and 3:5-dichlorocatechol by Messrs Monsanto Chemical Company; 4-chlorocatechol by Professor R. T. Williams. Other substances were obtained commercially.

RESULTS

Effect of age on activity of adapted cells

The *Achromobacter* strain was grown on agar plates containing MCPA and after 3, 6 and 10 days of incubation, respectively, batches of organisms were harvested, washed in buffer solution and used for manometric experiments. The rates of oxygen uptake by organisms from cultures of different ages were

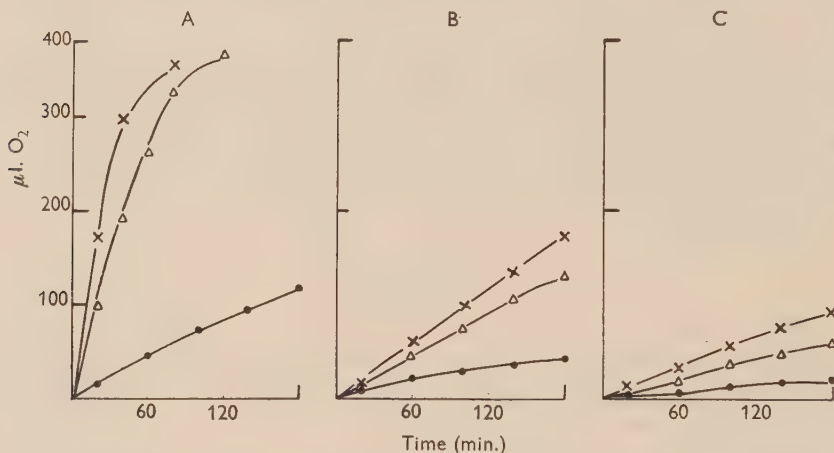


Fig. 1. Comparison of MCPA-grown *Achromobacter* organisms from cultures of different ages. Oxygen uptake by washed organisms (●—●) alone and in the presence of 2 μ mole MCPA/3 ml. (Δ — Δ) or 2 μ mole 2:4-D/3 ml. (\times — \times). A, 3-day cultures; B, 6-day cultures; C, 10-day cultures.

determined in the presence of 2 μ mole/3 ml. of 2:4-D and MCPA as substrates. The results, plotted in Fig. 1, show that organisms from 3-day cultures completed the oxidation of the substrate in about 1½ hr. whereas less than 50% oxidation occurred, even after 4 hr., when using organisms from 6- or 10-day cultures. Similar results (Fig. 2) were obtained with *Flavobacterium peregrinum*, (Stapp & Spicher, 1954) grown on 2:4-D-containing medium. The importance of using only young cultures in manometric experiments with these organisms was thus demonstrated.

Oxygen uptake experiments with isomers of 2:4-D

Washed organisms from 3-day cultures of the *Achromobacter* strain, grown on 2:4-D medium, were used to test the five isomeric compounds 2:3-, 2:5-, 2:6-, 3:4- and 3:5-dichlorophenoxyacetic acids as substrates in an experiment to measure rates of oxygen uptake. None of these compounds was oxidized by the 2:4-D-adapted bacteria.

In a similar experiment, but using 2:4-D-grown organisms of the *Flavobacterium peregrinum* strain, none of the isomers of 2:4-D was oxidized to any significant extent, although in some cases the rates of oxygen uptake were slightly above the endogenous rate (see Fig. 3). It was evident, therefore, that

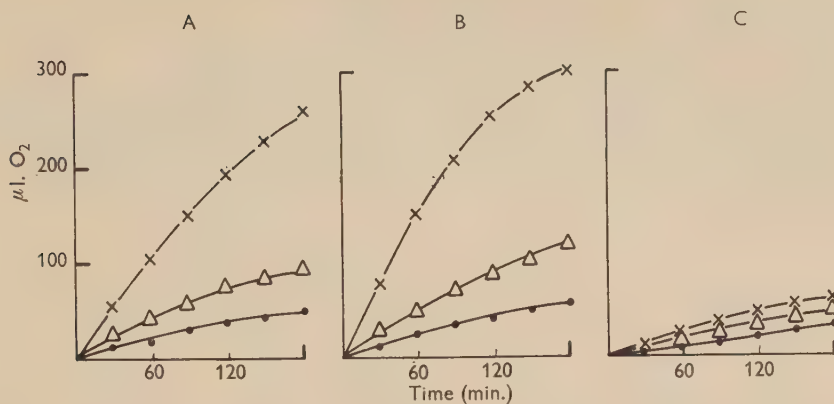


Fig. 2. Comparison of 2:4-D-grown *Flavobacterium peregrinum* organisms from cultures of different ages. Oxygen uptake by washed organisms alone (●—●) and in the presence of 2 μ mole MCPA/3 ml. (Δ — Δ) or 2 μ mole 2:4-D/3 ml. (x—x); A, from 3-day cultures; B, from 5-day cultures; C, from 9-day cultures.

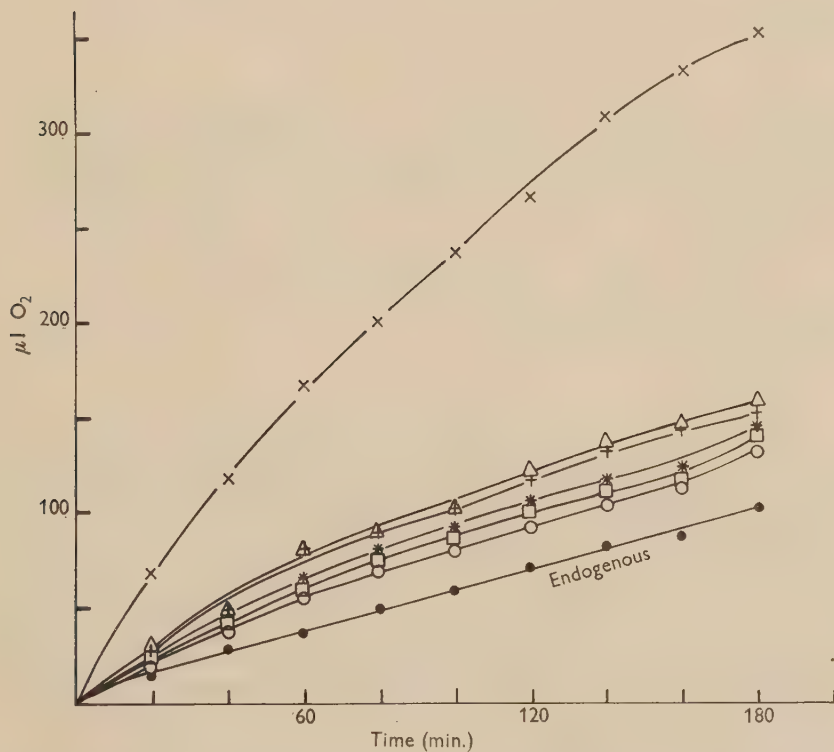


Fig. 3. Rate of oxygen uptake by washed, 2:4-D-grown *Flavobacterium peregrinum* organisms alone (●—●) and in the presence of 2 μ mole/3 ml. 2:3-dichloro- (Δ — Δ), 2:4-dichloro- (x—x), 2:5-dichloro- (+—+), 2:6-dichloro- (O—O), 3:4-dichloro- (*—*) and 3:5-dichlorophenoxyacetic acid (\square — \square) as substrate.

growth in the presence of 2:4-D resulted in organisms with a specific adaptation to metabolize 2:4-D and none of the isomers.

Oxidation of mono-chlorophenoxyacetic acids

Rates of oxygen uptake were measured for washed *Achromobacter* organisms which had been grown on 2:4-D medium, with 2-, 3- or 4-chlorophenoxyacetic acid as substrate. The results (Fig. 4) show that 4-chlorophenoxyacetic acid

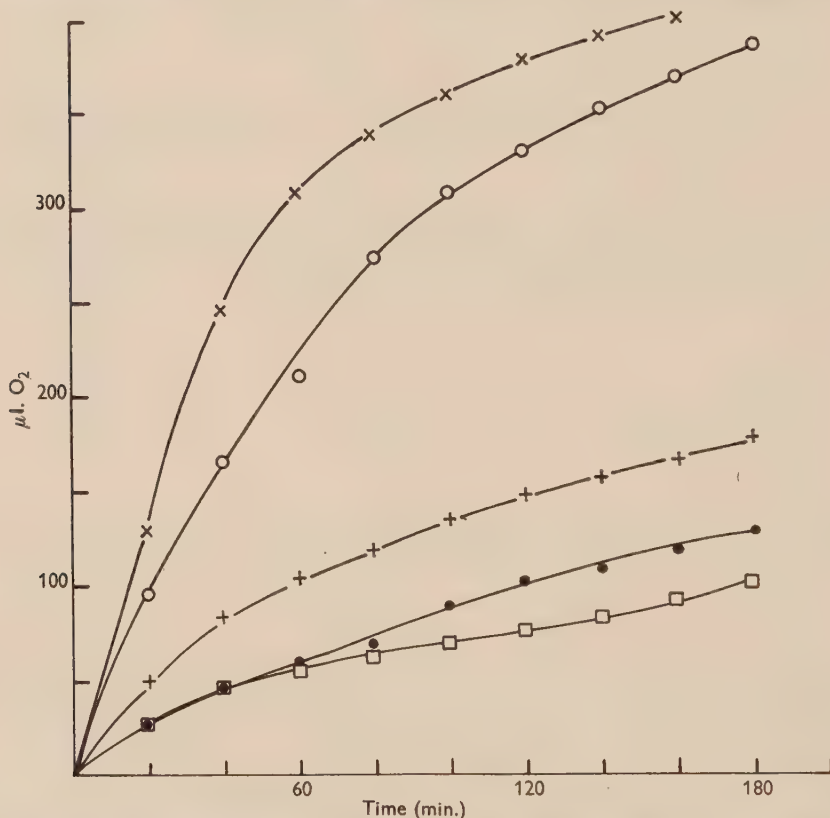


Fig. 4. Rate of oxygen uptake by *Achromobacter* organisms grown on 2:4-D. In presence of 2 μ mole/3 ml. 2-chloro- (+—+), 3-chloro- (□—□), 4-chloro- (○—○) and 2:4-dichlorophenoxyacetic acid (×—×) as substrate.

was oxidized at nearly the same rate as 2:4-D; 2-chlorophenoxyacetic acid was oxidized at a slower rate and to a much lesser extent, but the 3-chloro-compound was not oxidized and even depressed slightly the endogenous respiration. Similar results were obtained with washed *Flavobacterium peregrinum* organisms which had been grown with 2:4-D.

Cross-adaptation experiments

Since, when grown on MCPA medium, the *Achromobacter* strain was adapted to oxidize both MCPA and 2:4-D, an experiment was made to determine whether 2:4-D-adapted organisms could also oxidize MCPA. A culture of

the *Achromobacter* strain was grown on 2:4-D medium and then a series of three successive transfers on to fresh media after every 2 or 3 days was made; samples of organisms from each transfer were collected separately and washed. Rates of oxygen uptake by these organisms, with 2:4-D or MCPA as substrate, were determined in the usual manner. The results (Fig. 5) showed that after

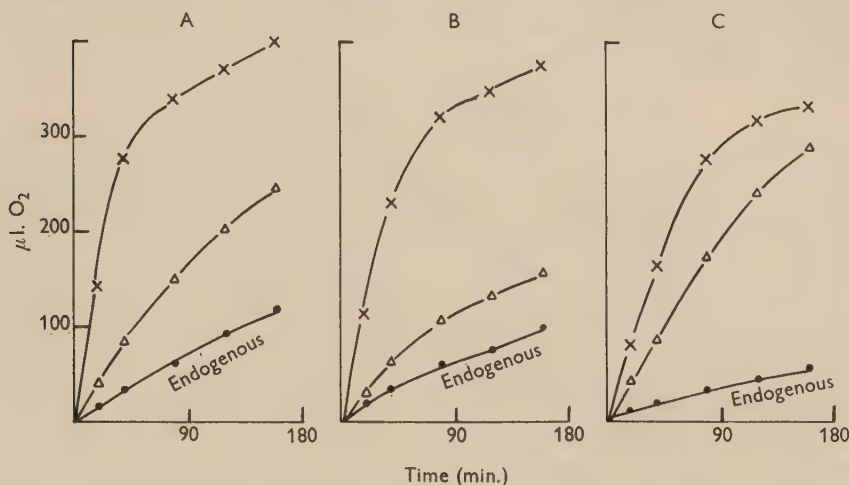


Fig. 5. Effect of successive transfers on 2:4-D medium on the rate of oxygen uptake by *Achromobacter* organisms compared with MCPA-grown organisms: in the presence of $2\text{ }\mu\text{mole/3 ml.}$ 2:4-D (\times — \times) or MCPA (Δ — Δ) as substrate. A, Organisms after one transfer on 2:4-D medium. B, Organisms after three transfers on 2:4-D medium. C, Organisms grown on MCPA after three previous subcultures on 2:4-D medium.

three transfers on 2:4-D medium, the organisms oxidized MCPA at a much slower rate than when first grown on 2:4-D; but when they were again grown on MCPA medium, the organisms recovered their former rate of MCPA oxidation.

Oxidation of bromo-derivatives of phenoxyacetic acid

The above results indicated that position isomerism was an important factor in determining the specificity of the adaptive enzymes of these organisms. It seemed of interest, therefore, to find whether substitution of chlorine by bromine would affect the susceptibility to oxidation of the substituted phenoxyacetic acid. Experiments showed that 2:4-D-grown organisms of either strain and MCPA-grown *Achromobacter* organisms all oxidized 2:4-dibromophenoxyacetic acid as fast as they did 2:4-D. 4-Bromo-2-chlorophenoxyacetic acid was oxidized similarly by 2:4-D-grown *Achromobacter* organisms.

2:4-Dibromophenoxyacetic acid could replace 2:4-D in media for the growth of either strain, so some experiments were carried out with *Achromobacter* organisms grown on the dibromo compound. The results obtained were similar to those given by 2:4-D-grown organisms (see Fig. 5B) in that the rate of oxygen uptake with 2:4-dibromo- or 2:4-dichlorophenoxyacetic acid was about the same, and with MCPA the rate of oxidation was much slower.

Since it has been shown that 2:4-D- or MCPA-adapted bacteria oxidize 4-chloro-, 2:4-dichloro-, 4-chloro-2-methyl-, 4-bromo-2-chloro-, 2:4-dibromo-, and to some extent, 2-chlorophenoxyacetic acid, the conditions which determine the susceptibility of a mono- or di-substituted phenoxyacetic acid to oxidation may be summarized as follows: the first substituent should be a halogen and should occupy position 4 or 2; the second substituent in a 4-substituted compound should be in position 2 and may be either halogen or a methyl group.

Oxidation of possible intermediate metabolites

In earlier work, Steenson & Walker (1956) failed to demonstrate the oxidation of 6-hydroxy-2:4-dichlorophenoxyacetic acid or of 2:4-dichlorophenol by 2:4-D-adapted bacteria; but since finding that organisms from young cultures were much more active in respiring on 2:4-D, it became important to re-examine the effect of such organisms on these compounds. The effect of substrate concentration, especially of antiseptic phenols, may also be critical; e.g. phenol is catabolized by certain bacteria at concentrations below 0.01 M, but above this it acts as a general antiseptic. Dichlorophenol is a stronger antiseptic than phenol, so it was necessary to examine the effect of this compound at low concentrations.

6-Hydroxy-2:4-dichlorophenoxyacetic acid. In experiments with washed organisms of either strain, grown on 2:4-D medium for not more than 3 days, no oxygen uptake was observed with 6-hydroxy-2:4-dichlorophenoxyacetic acid as substrate. With the *Achromobacter* strain, grown on MCPA medium for 3 days, there was no oxygen uptake, but the endogenous respiration was slightly depressed by 6-hydroxy-2:4-dichlorophenoxyacetic acid. Thus no evidence that the latter compound might be a metabolite of 2:4-D was obtained.

2:4-Dichlorophenol. Fig. 6 shows the results of an experiment on the oxidation of 2:4-dichlorophenol by 2:4-D-grown *Achromobacter* organisms. One μ mole 2:4-dichlorophenol/3 ml. was oxidized immediately at the same rate as 2:4-D, but with 2 μ mole/3 ml. there was a moderate lag period before the rate of oxygen uptake reached that of 2:4-D. In another experiment it was found that 1.5 μ mole 2:4-dichlorophenol/3 ml. was oxidized immediately, but with 2 μ mole/3 ml. a lag period was again observed, indicating a toxic concentration. Peptone-grown *Achromobacter* organisms did not oxidize 2:4-dichlorophenol at a concentration of 1 μ mole/3 ml. The toxic effect of 2:4-dichlorophenol was demonstrated also in an experiment in which the oxidation by adapted organisms of 2 μ mole 2:4-D/3 ml. and a mixture of 2 μ mole 2:4-D + 2 μ mole 2:4-dichlorophenol/3 ml. was compared. The results (Fig. 7) showed that there was a lag period before the mixed substrates were oxidized.

On the basis of Stanier's simultaneous adaptation hypothesis, therefore, it would appear that 2:4-dichlorophenol is an intermediary metabolite in 2:4-D dissimilation, the early stages of which involve the loss by oxidation of the acetic acid residue. It was therefore of interest to determine whether the phenols, at suitable concentrations, corresponding to the other phenoxyacetic

acid derivatives subject to bacterial decomposition were oxidized immediately. *Achromobacter* organisms grown on 2:4-D oxidized 2:4-dibromophenol at a concentration not exceeding $M/3000$ ($M/1500$ was toxic), and 4-chlorophenol at $M/3000$ ($M/1500$ was slightly toxic). Neither phenol nor 2-chlorophenol was oxidized. MCPA-grown *Achromobacter* organisms oxidized 5-chloro-2-cresol at $M/6000$ and, with a slight lag, at $M/3000$; 2:4-dichlorophenol at $M/1500$; 2:4-dibromophenol at $M/3000$; but not 2- or 4-cresol. Peptone-grown *Achromobacter* organisms did not oxidize $M/3000$ 5-chloro-2-cresol.

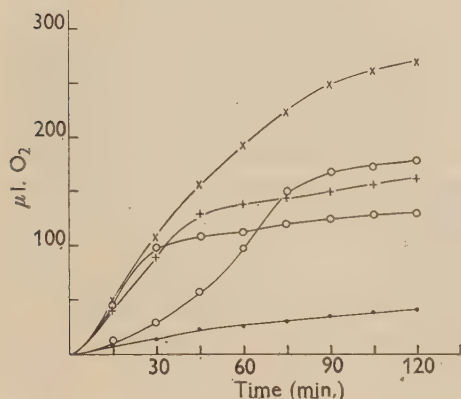


Fig. 6

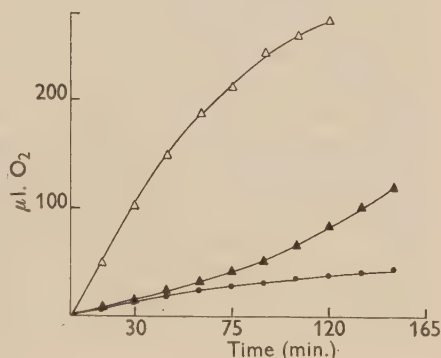


Fig. 7

Fig. 6. Rate of oxygen uptake by washed *Achromobacter* organisms (●—●), grown on 2:4-D medium, and in the presence of 2 μ mole 2:4-D/3 ml. (x—x), 2 μ mole 4-chlorocatechol/3 ml. (+—+) and 1 and 2 μ mole 2:4-dichlorophenol/3 ml. (○—○).

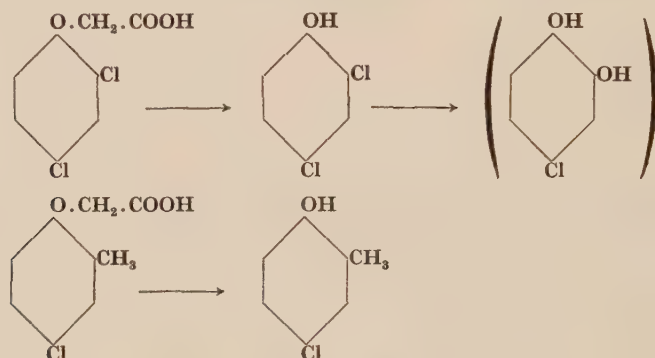
Fig. 7. Effect of 2:4-D-grown *Achromobacter* organisms on 2:4-D in the presence of 2:4-dichlorophenol. Oxygen uptake by washed organisms alone (●—●) and in the presence of 2 μ mole 2:4-D/3 ml. (Δ—Δ) and a mixture (▲—▲) of 2:4-D (2 μ mole) and 2:4-dichlorophenol (2 μ mole).

4-Chlorocatechol. Washed *Achromobacter* organisms, grown on 2:4-D or MCPA media, oxidized 4-chlorocatechol immediately at the same rate as 2:4-D (Fig. 6). No oxygen uptake was observed with 2-hydroxy-4-chlorophenoxyacetic acid or 3:5-dichlorocatechol, but catechol was oxidized by 2:4-D-adapted organisms. Neither catechol nor 4-chlorocatechol was oxidized by peptone-grown organisms. After mixing the adapted organisms with catechol or 4-chlorocatechol, a deep yellow colour developed fairly quickly. The colour was discharged by acidifying with dilute aqueous hydrochloric acid and on shaking with ether, separating the ethereal layer and then extracting the latter with aqueous sodium hydrogen carbonate, the yellow coloured material was recovered. The yellow material showed similar behaviour therefore to the yellow acidic substance detected by us (Stenson & Walker, 1956) in cultures of either strain growing in a liquid medium containing 4-chlorophenoxyacetic acid. The colour formed in the Warburg vessels was more intense than that in liquid cultures of 4-chlorophenoxyacetic acid medium. In similar manometric experiments with MCPA-grown organisms and 4-chlorophenol as substrate, a slow development of a pale yellow colour was observed,

but it was much less intense than with 4-chlorocatechol. It seems possible that 4-chlorophenol may be oxidized through 4-chlorocatechol which subsequently gives rise to the yellow substance.

DISCUSSION

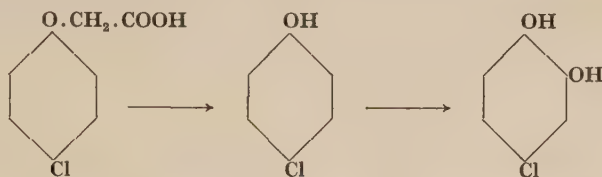
It is desirable to use as many criteria as possible in deciding whether a given compound is an intermediate in the bacterial dissimilation of an initial substrate; e.g. the detection of the supposed intermediate in cultures, conformity with Stanier's simultaneous adaptation hypothesis and the ability of the intermediate to support growth of the organism. Audus & Symonds (1955) commented on the difficulties of growing adapted *Bacterium globiforme* in mineral salts + 2:4-D solution but they studied the growth rates of the organism and followed the rate of loss of phytotoxicity in cultures. They attempted to isolate intermediates by ether extraction of acidified cultures, but did not identify them. Our results from manometric experiments with adapted organisms of two bacterial species suggest the following scheme for the early stages in the metabolism of 2:4-D and MCPA:



The first step seems to be the oxidation of the acetic acid moiety of the substituted phenoxyacetic acid to give the corresponding phenol, viz. 2:4-dichlorophenol and 5-chloro-2-cresol respectively. It is likely that 2:4-dichlorophenol is converted next to 4-chlorocatechol, although the evidence is rendered less decisive by the fact that adapted organisms can oxidize either catechol or 4-chlorocatechol. The failure to oxidize 3:5-dichlorocatechol is further support for the rejection of 6-hydroxy-2:4-dichlorophenoxyacetic acid as an intermediate. More evidence is required, however, to establish the precise reactions by which the chlorine atom is lost from the dichlorophenol.

The above pathway is not analogous to that which operates with 4-chlorophenoxyacetic acid, which Evans & Smith (1954) found to be dissimilated through 4-chloro-2-hydroxyphenoxyacetic acid and 4-chlorocatechol. With 2:4-D metabolism, there is no evidence that 6-hydroxy-2:4-D is involved. It is interesting that when 2:4-D adapted organisms of our strains are inoculated into 4-chlorophenoxyacetate media, and incubated for 2 or 3 days, there is formed a yellow substance which appears to be identical with that formed by the action of suspensions of these organisms on 4-chlorocatechol or, to a smaller

extent, on 4-chlorophenol. The same organisms do not oxidize 4-chloro-2-hydroxyphenoxyacetic acid, so it seems likely that our 2:4-D-adapted organisms oxidize 4-chlorophenoxyacetic acid to 4-chlorocatechol via 4-chlorophenol and not through 4-chloro-2-hydroxyphenoxyacetic acid:



Whether the yellow substance is in fact 4-chloro-*o*-benzoquinone, or is derived from it, has not been established.

Rogoff & Reid (1956) showed that 2:4-D-adapted organisms of a *Corynebacterium* sp. from soil could oxidize 2:4-dichlorophenol. Audus (1952) suggested that this compound might be an intermediate breakdown product of 2:4-D because he observed a rapid disappearance of 2:4-dichlorophenol when a dilute solution was perfused through soil crumbs previously enriched with 2:4-D. He noted also the toxic effect of an excess of 2:4-dichlorophenol. These findings are in agreement with our results obtained with pure cultures of bacteria and from the behaviour of washed bacteria in manometric experiments. Further study of the decomposition of 2:4-dichlorophenol and 5-chloro-2-cresol by adapted bacteria is desirable.

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Physiological Factors in the Production of an Iodophilic Polysaccharide from Pentose by a Sheep Rumen Bacterium

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SUMMARY: A Gram-negative, non-flagellated, asporogenous, anaerobic, iodophilic, curved rod ($2.5 \mu. \times 0.5 \mu.$) was isolated from the rumen of a sheep and designated *Bacteroides amylogenes* n.sp. This organism transforms a number of carbohydrates including pentoses to intracellular iodophilic polysaccharide which has been isolated and shown to be a polymer of glucose. Resting suspensions of this organism utilize this iodophilic material as a reserve substance under a variety of conditions. *Bacteroides amylogenes* grows optimally *in vitro* when bicarbonate and rumen liquor are included in the medium. On physiological and morphological grounds it is considered to be an authentic rumen bacterium. The chief fermentation product from xylose is butyric acid.

Recent investigations by Robinson, Doetsch, Sirotnak & Shaw (1955), Mann & Oxford (1955), Gibbons, Doetsch & Shaw (1955), and Hobson & Mann (1955) revealed that authentic rumen bacteria may produce intracellular iodophilic polysaccharide from carbohydrate substrates. Earlier references dealing with 'starch' or 'glycogen' formation in bacteria of avian or mammalian intestines are given in Hobson & Mann (1955). Heretofore there has been but one report concerning iodophilic polysaccharide formation from D (+) xylose by mixed suspensions of bovine rumen bacteria (Gibbons *et al.* 1955). It was shown that a polysaccharide 'ISS' (an abbreviation for iodine staining substance), composed solely of glucose, was produced from either D (+) glucose or D (+) xylose by a mixed rumen bacterial flora, irrespective of the number of different 'species' that might be present.

The present paper studies the physiological factors which govern this interesting polymerization phenomenon when a pure culture of a Gram-negative sheep rumen bacterium, not previously described, is used. In view of this, morphological, physiological and nutritional data are given for taxonomic characterization of the organism. Although on morphological grounds alone (see organism no. 9 (fig. 21) of Moir & Masson, 1952, which resembles it) it may well be a rumen bacterium, the authenticity of this isolate is judged mainly in the physiological sense, i.e. it is obligately anaerobic; grows only over a narrow temperature range near 39°; requires a factor(s) found in rumen liquor for optimal *in vitro* growth; and forms ISS and end products normally associated with rumen liquor.

METHODS

Isolation of xylose-fermenting bacteria

Rumen liquor samples were obtained from fistulated sheep no. 879 (Eadie & Oxford, 1955). This animal was fed 750 g. hay and 450 g. concentrates/day. Rumen liquor samples were taken *c.* 3 hr. after morning feeding. The culture technique of Hungate (1950) was used, but with the dilution blanks devised by Doetsch, Robinson & Shaw (1952). D (+) Xylose (substituted for D (+) glucose and cellobiose) cysteine hydrochloride and Na₂CO₃ were sterilized together by Seitz filtration and added aseptically to the autoclaved rumen liquor + mineral salts agar to give final concentrations (w/v) of 1, 0.1 and 0.4 %, respectively. Roll tubes were incubated at 39° and colonies selected from 10⁻⁷ or 10⁻⁸ dilutions after 48–72 hr. Colonies were subcultured only when microscopic examination of a wet mount stained with Lugol's iodine solution revealed a purple or blue coloration within the cells (Hobson & Mann, 1955). One isolate (4.13) was selected from a collection of xylose-fermenting rumen bacteria for intensive study. Conventional pure culture techniques were used for Gram's staining method, capsule stain, granule stain, indole, H₂S and catalase production, and optimum growth temperature. Motility determinations were made in sealed capillary tubes, and flagella stains were made by the methods of Fisher & Conn (1942), Leifson (1951) and Plimmer & Paine (1921).

Range of compounds transformed to iodophilic polysaccharide

Most of the compounds studied (Table 1) were sterilized by Seitz filtration and substituted in final concentration of 1 % (w/v) for the D (+) xylose in the isolation medium. Agar slopes were streaked and stabbed with one loopful of a 24 hr. culture of organism 4.13 grown on xylose-rumen liquor + mineral salts agar (hereafter referred to as 'X agar'). Controls were X agar itself and the medium without added D (+) xylose. As soon as 'fair' (1+) growth appeared, the organism was subcultured to a fresh tube of the same medium to obviate 'carry over' effects. In no instance did growth appear in the medium without carbohydrate. Density of growth was estimated as 'fair' (1+) to 'luxuriant' (4+). Immediately following subculture the initial slope was flooded with Lugol's iodine solution and the colour noted. It was considered necessary to do so at the earliest possible moment after growth appeared, since the iodophilic polysaccharide is a reserve substance and is utilized upon continued incubation. Acid, but not necessarily gas formation was detected in this study, since carbonate (which appeared essential for optimal growth) liberated as CO₂ by fermentation acids split the agar and produced frothiness in the syneresis water.

Nutritional requirements

Several workers (Hungate, 1950; McNeill, Doetsch & Shaw, 1954; Bryant & Doetsch, 1954*a*) have shown that factors(s) found in rumen liquor are essential for *in vitro* cultivation of many authentic rumen bacteria. In addition to studying this point with isolate 4.13, the effect of bicarbonate on growth was

Table 1. *Growth and 'starch' production by Bacteroides amylogenes on various substrates*

The concentration of substrate in the agar medium was 1 % (w/v) unless otherwise stated; it was sterilized separately by Seitz filtration of a concentrated aqueous solution except in the case of polysaccharides of high molecular weight when sterilization was by intermittent steaming. Analar carbohydrates were used wherever possible; the source of the rarer sugars is indicated below.

Substrate	Amount of bacterial growth in (n) days	Iodophilic reaction of growth
Aldopentoses		
D (+) Xylose	4+(1)	+
L (-) Xylose (L. Light)	0 (8)	0
D (-) Arabinose (L. Light)	1+(1)	+
L (+) Arabinose (Kerfoot)	4+(1)	+
D (+) Ribose (L. Light)	1+(2)	+
D (+) Lyxose (L. Light)	0 (5)	0
Methylaldopentoses		
L (+) Rhamnose	4+(1)	+
L (-) Fucose (L. Light)	4+(1)	+
Aldohexoses		
D (+) Glucose	4+(1)	+
D (+) Mannose (recrystallized from ethanol and methanol)	4+(1)	+
D (+) Galactose	4+(1)	+
Ketohexoses		
D (-) Fructose	4+(1)	+
Sorbose	0 (8)	0
Sugar alcohols		
Glycerol	1+(4)	±
Adonitol (G. T. Gurr)	0 (8)	0
Mannitol	0 (6)	0
Sorbitol	0 (6)	0
Dulcitol	0 (8)	0
Inositol	0 (4)	0
Aminosugar		
Glucosamine hydrochloride	4+(2)	+
Hexose phosphates (0.5 %, w/v)		
Glucose-1-phosphate (K salt)	1+(5)	+
Glucose-6-phosphate (K salt)	1+(2)	+
Disaccharides		
Sucrose	4+(1)	+
Maltose	4+(1)	+
Lactose	1+(4)	+
Cellobiose	4+(1)	+
Trehalose (G. T. Gurr)	4+(1)	+
Melibiose (L. Light)	4+(1)	+
Trisaccharide		
Raffinose (Kerfoot)	4+(1)	+
Glycosides		
α-Methylglucoside (B.D.H.)	0 (8)	0
Salicin	4+(1)	+
Aesculin (G. T. Gurr)	4+(1)	+
Pentosans		
Soluble wheat flour pentosan entirely free from hexosan; (contains xylose and arabinose units, Perlin, 1951)	3+(1)	+
Soluble xylan of <i>Rhodymenia palmata</i> (Barry & Dillon, 1940)	1+(2)	+

Table 1 (cont.)

Hexosans		
Inulin (Kerfoot)	4+(1)	+
Dextrin (Kerfoot)	1+(1)	+
Glycogen	0 (6)	0
Amylose, (0.5 %, w/v)	0 (6)	0
Holotrich starch (amylopectin) (Forsyth & Hirst, 1953)	0 (6)	0
0.5 % (w/v)		
Soluble starch (B.D.H.)	0 (6)	0
Cotton cellulose (finely divided, 0.2 %, w/v)	0 (12)	0
<i>Bacteroides amylogenes</i> 'starch'	0 (6)	0

Organic acids

The organism did not utilize any of the following: glucuronate, pyruvate, lactate, fumarate, succinate, citrate.

investigated. Substitutions for 40 % (w/v) rumen liquor in X agar (all given as % (w/v) 'Bacto' products) were made as follows: (1) 0.5 % Casitone; (2) 0.5 % Casitone + 0.5 % yeast extract; (3) 0.5 % yeast extract; (4) 0.5 % Tryptose; (5) 0.5 % yeast extract + 0.5 % peptone; (6) same as (1) but 0.066 M-phosphate buffer (pH 7.0) added aseptically (30 ml. in 50 ml. medium) without Na_2CO_3 , and tubed under N_2 ; (7) X agar without Na_2CO_3 and tubed under N_2 ; (8) a defined medium composed of the following per 100 ml.: calcium-D-pantothenate, 0.1 mg.; nicotinic acid, 0.25 mg.; pteroylglutamic acid, 0.001 mg.; thiamine hydrochloride, *p*-aminobenzoic acid, pyridoxine hydrochloride and riboflavin, 0.1 mg. each; inositol, choline chloride, nicotinamide, uracil, guanine hydrochloride, adenine sulphate and xanthine hydrochloride 0.5 mg. each; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.15 mg. each; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.014 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.056 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0034 g.; *n*-valeric acid, 5.0 mg.; *iso*-valeric acid, 2.5 mg.; sodium acetate, 0.41 g., sodium citrate $\cdot 2\text{H}_2\text{O}$, 0.2 g., NH_4Cl , 0.152 g., resazurin, 0.5 ml. of a 0.1 % (w/v) aqueous solution; agar (Gurr) 1.5 g., D (+) xylose, 1.0 g.; cysteine hydrochloride, 0.1 g.; Na_2CO_3 , 0.4 g. The latter three compounds were Seitz filtered and added aseptically to the medium which was then tubed under CO_2 ; (9) same as (8) but with 0.5 % each of yeast extract and proteose-peptone added. The density of growth was compared with that on X agar after 24 and 48 hr. of incubation at 39°, and for longer periods when necessary. Four serial subcultures were made on each medium supporting growth of isolate 4-13 before the observation was recorded.

Factors influencing the utilization of iodophilic polysaccharide

The growth of isolate 4-13 from 35 tubes of X agar (44 hr. at 39°) was washed off with 0.066 M-phosphate buffer (pH 7.0). It was found necessary to grow the organism on slopes of solid medium after repeated failure of attempts to obtain good growth quickly in liquid media or on solid medium in Roux bottles. The organisms were washed twice in buffer (no reducing sugar in supernatant) and the optical density adjusted to 0.78 at 640 m μ . (EEL filter no. 205) with the EEL (Evans Electroselenium Ltd., Harlow, Essex) colorimeter. These suspensions had a protein nitrogen content of usually about

0.28 mg./ml. Suspensions of organisms, adjuncts and buffer were made to a volume of 3 ml. in small rubber-stoppered test tubes and incubated under the appropriate O_2 -free gas in a 39° water bath. Table 2 is a protocol of the various factors investigated. After 1, 3 and 5 hr. incubation 0.25 ml. samples were removed from each tube and the organisms sedimented with an M.S.E. angle centrifuge. The sediment was washed once in buffer and the organisms suspended in a final volume of 2 ml. One-tenth ml. iodine solution (aqueous solution of 2% (w/v) KI + 0.2% (w/v) I_2) was added with thorough mixing, and the optical density read on the EEL colorimeter against a boiled-cell control arbitrarily set at $O.D. = 1.00$. The rate of disappearance of the blue colour was taken as a measure of polysaccharide utilization and the limiting optical density upon depletion was between 0.5 and 0.6.

End products of xylose fermentation

Several workers (Heald, 1952; Doetsch, Robinson, Brown & Shaw, 1953; Howard, 1955) have observed that mixed suspensions of rumen bacteria do not produce lactic acid from pentoses. To determine whether this held for isolate 4.13, and to note whether fatty acids characteristic of rumen liquor were produced from D (+) xylose fermentation, a liquid medium was devised which allowed fairly good if slow growth. The medium had the following composition: 0.5% (w/v) yeast extract, 0.5% (w/v) proteose-peptone, 10% (v/v) strained rumen liquor, 7.5% (v/v) of each of Hungate's mineral salt solutions, 0.1% (v/v) of a 0.1% (w/v) aqueous resazurin solution, 1.0% (w/v) D (+) xylose, 0.1% (w/v) cysteine hydrochloride, and 0.4% (w/v) Na_2CO_3 . The latter three compounds were sterilized by Seitz filtration and added aseptically to the autoclaved medium. Inoculum was one ml. of the complete medium used to wash off the growth from a 24 hr. culture of isolate 4.13 on X agar. Duplicate flasks (150 ml. volume filled two-thirds full) were set up, one incubated for a week at 39° and the other stored at 2° . At the end of the incubation period residual carbohydrate was determined by the method of Somogyi (1945), lactic acid by the Barker & Summerson (1941) method, and fatty acids by the method of James & Martin (1952).

Purification and characterization of iodophilic polysaccharide

Growth from 500 slope cultures of isolate 4.13 grown for 44 hr. on X agar at 39° was washed off with 0.066 M-phosphate buffer (pH 7.0) and twice filtered through 1 in. layers of closely packed glass wool. The organisms were washed twice in buffer and suspended in a minimal (1–3 ml.) amount of buffer. Reducing sugars were absent from the supernatant fluids of these washings. The resulting bacterial paste was kept at -20° until the extraction process was begun. The method used was that of Hobson & Mann (1955).

Sixteen g. cell paste (wet wt.) were ground in a cold mortar with 32 g. cold, acid-washed, acetone-extracted carborundum (grade F) [Griffin & Tatlock, Ltd., London] for 15 min. and the mass twice extracted with 33% (w/v) chloral hydrate at 80° . The extract was filtered through a sintered glass filter

(grade 5) and the filtrate slowly added with stirring to 4 vol. cold acetone. After standing 24 hr. at 2° the precipitated polysaccharide was collected by centrifugation and dried by trituration first with acetone and then with ether (yield 320 mg.). One hundred mg. of crude polysaccharide powder was wetted with ethanol and dissolved in 0.1 N-NaOH by heating in a boiling water bath for several minutes. The resulting suspension was neutralized to the phenolphthalein end point and to 50 ml. of liquor was added 2 g. hydrated sodium acetate, 10 ml. chloroform and 1 ml. *n*-butanol. After shaking for 10 min. in a separating funnel, the aqueous layer was removed and dialysed against running distilled water for 3 days. The non-diffusing polysaccharide was precipitated by 2 vol. cold ethanol, allowed to stand for 24 hr. at 2°, centrifuged and dried by grinding in ethanol and finally in ether. The resulting powder was dried *in vacuo* over P₂O₅. The purified polysaccharide (3.5 mg.) was hydrolysed in 0.5 ml. of 2N-H₂SO₄ in a sealed tube at 100° for 2 hr. and neutralized with BaCO₃. Constituent monosaccharides were detected by descending paper chromatography using: (a) *n*-butanol:pyridine:water (10:3:3); (b) ethyl acetate:pyridine:water (10:4:3); (c) *n*-butanol:glacial acetic acid:water (4:1:5). Aniline phosphate (0.05 M) was the developing agent in each instance. Reference carbohydrates were D (+) glucose, D (+) xylose, L (+) arabinose, D (+) galactose and L (+) rhamnose. 'Starch' content was assayed by estimating reducing sugar as glucose (Somogyi, 1945), blue value was determined by the method of Hobson & MacPherson (1952), ash by ignition, and total nitrogen by the micro-Kjeldahl method.

RESULTS

Characterization of the organism

Isolate 4.13 is a Gram-negative curved rod most closely allied to *Bacteroides*. Hereafter it will be referred to as *Bacteroides amylogenes* n.sp., whose characteristics are the following:

Bacteroides amylogenes n.sp. is a Gram-negative, non-motile, non-flagellated, asporogenous, non-capsulated, anaerobic, iodophilic, curved rod (2.5 μ . long by 0.5 μ . wide) from the rumen of a sheep (Pl. 1, fig. 1). It occurs singly and in short chains of organisms superficially resembling spirilla. The organisms are quite fragile and crush easily when pressed between glass slides. The organism is mesophilic and grows best at 39°, slowly at 32°, and not at all at 22° or 55°. Colonies on X agar are small (1–2 mm.), circular, raised, mucoid and pearl white. Twenty-four to forty-eight hr. colonies stain purple to blue black when covered with Lugol's iodine solution (Pl. 1, fig. 2) and organisms from these colonies are uniformly purple (Pl. 1, fig. 3). Rumen liquor or other complex nitrogenous nutrient, bicarbonate and a fermentable carbohydrate are needed for good *in vitro* growth. The organism ferments D (+) xylose, L (+) arabinose, L (+) rhamnose, L (–) fucose, D (+) glucose, D (+) mannose, D (+) galactose, D (–) fructose, glucosamine hydrochloride, sucrose, maltose, lactose, cellobiose, trehalose, melibiose, raffinose, salicin, aesculin and soluble pentosans with the production of acid and an intracellular iodophilic poly-

saccharide. It does not ferment L (−) xylose, D (+) lyxose, sorbose, adonitol, sorbitol, mannitol, dulcitol, inositol, α -methyl-glucoside, glycogen, amylose, soluble starch or cellulose. H₂S is produced from cysteine hydrochloride. Indole not formed. Catalase-negative.

Although *Bacteroides amylogenes* possesses a distinct cell wall when stained by Robinow's (1945) method, it appeared to bend and flex to and fro when observed in capillary tube preparations. This motion did not result in motility or physical progress as far as could be determined. The organism is obligately anaerobic and fails to grow in the absence of suitable reducing agents (as 0.1 % (w/v) cysteine hydrochloride), or when the resazurin indicator is pink to any degree. It survived 5 days of exposure to atmospheric oxygen, and subcultures so treated grew readily upon incubation under anaerobic conditions. Examination of stained preparations made from tubes in which the resazurin indicator showed a trace of oxygen present revealed long festoons and strands of organisms, many possessing 'bulbs' (Pl. 1, fig. 4) typical of those described by Hughes (1956). Probably these are pathological manifestations elicited by growth in the presence of inhibitory amounts of oxygen. Subculture and incubation of these forms under anaerobic conditions resulted in cells of 'regular' morphology.

Polysaccharide formation and utilization studies

The data in Table 1 reveal that a number of compounds, all carbohydrates, are transformed to iodophilic polysaccharide. It should be emphasized that in every case of growth iodophilic polysaccharide was produced. Notable among compounds not supporting growth were sugar alcohols, dicarboxylic acids, and hexosans except inulin, and to a lesser extent, dextrin. Soluble pentosans were readily fermented, whereas holotrich starch (Forsyth & Hirst, 1953) and purified *Bacteroides amylogenes* polysaccharide were not fermented. The pentoses are of some interest, particularly since C₅ units must be degraded and resynthesized to the C₆ units of the polysaccharide (see below). D (+) Lyxose, a pentose not found in nature, and L (−) xylose were not fermented; and D (−) arabinose and D (+) ribose supported only fair (1+) growth, yet these colonies were intensely iodophilic.

Bacteroides amylogenes grew poorly (2+ at best) on the first eight media listed under 'nutritional requirements'. Medium 9 supported growth equal to that obtained on X agar. It is significant that this organism did not grow well (2+) on X agar without bicarbonate (medium 7), and not at all on defined medium 8.

In Table 2 is shown the result of a typical experiment on the effect of various factors on the utilization of stored iodophilic polysaccharide by *Bacteroides amylogenes*. It was found to be readily utilized in 0.066 M-phosphate buffer (pH 7.0) under N₂, and the addition of 1 % (w/v) D (+) xylose or D (+) glucose did not retard the process. In fact, after 5 hr. at least 95 % of the added carbohydrate remained unused. Utilization was virtually nil in 0.066 M-phosphate buffer at pH 5.0 under N₂, or in 'tris' (tris-(hydroxymethyl)-aminomethane), pH 7.3, under N₂, and it was noticeably retarded

in 0.04 M-carbonate + bicarbonate buffer (pH 7.0) in air. Addition of Casitone, acid hydrolysed casein, or strained rumen liquor was without effect, and there was no appreciable depletion of polysaccharide in the boiled cell preparation during 5 hr.

Table 2. *Utilization of iodophilic polysaccharide under various test conditions*

Each tube had a final volume of 3.0 ml., including cell suspension (0.28 mg. N/ml.) adjunct and buffer under appropriate O₂-free atmosphere at 39°. All zero hr. readings were o.d. = 1.00 at 640 mμ.

Buffer	pH	Atmo- sphere	Adjunct or treatment	EEL readings after (n) hr.		
				(1)	(3)	(5)
0.04 M-HCO ₃ ⁻	7.0	CO ₂	—	0.95	0.90	0.87
0.1 M 'tris'	7.3	N ₂	—	0.97	0.96	0.95
0.066 M-PO ₄	5.0	N ₂	—	0.97	0.97	0.97
0.066 M-PO ₄	7.0	N ₂	Cells boiled 3 min.	0.98	0.98	0.97
0.066 M-PO ₄	7.0	Air	—	0.98	0.94	0.92
		N ₂	0.3 % (w/v) Casitone	—	0.74	0.70
		N ₂	0.3 % (w/v) acid hydrolysed casein	—	0.72	0.71
		N ₂	0.3 % (w/v) strained rumen liquor	—	0.72	0.72
		N ₂	1.0 % (w/v) D (+) xylose	0.87	0.71	0.61
		N ₂	1.0 % (w/v) D (+) glucose	0.87	0.68	0.68
		N ₂	—	0.86	0.77	0.67

Xylose fermentation and polysaccharide identification

In Table 3 are shown the products of D (+) xylose fermentation by *Bacteroides amylogenes*. These results indicate that 1.7 % of the carbon fermented can be accounted for in lactic acid, and 89 % in the volatile fatty acid fraction

Table 3. *Products of xylose fermentation by Bacteroides amylogenes*

	Acetic	Propionic	Butyric	Lactic
mmole/l.	18.6	5.3	38.6	1.3
% C of xylose used (46.3 mmole/l.)	16.1	6.9	66.7	1.7
	91.4 %			

the chief constituent of which is butyric acid. A typical analysis of the intracellular polysaccharide on a dry weight basis was: $[\alpha]_D^{28} = +178^\circ$ ($c = 0.432$ in 50 % (w/v) CaCl₂ solution); 'starch' content, 89 % after acid hydrolysis; blue value 11.6 colorimeter units; total nitrogen, 0.1 %; ash nil. The only sugar found in the acid hydrolysate by paper chromatography was glucose. At pH 6 and 37° the extracted polysaccharide was readily hydrolysed by the freeze-dried bacterial α -amylase prepared from a rumen streptococcus by Hobson & MacPherson (1952).

DISCUSSION

Species of the genus *Bacteroides* are not uncommonly found associated with the intestinal tract of mammals. Hungate (1950) and Bryant & Burkey (1953) described *B. succinogenes* as an important cellulolytic organism of the bovine rumen. In so doing, the characterization of the genus *Bacteroides*, hitherto described mainly in terms of pathological processes and medical diagnostic bacteriology (*Bergey's Manual*, 1948), was extended to include cellulolysis and the production of characteristic end products therefrom. Additional work by Bryant & Doetsch (1954*a, b*) established some of the nutritional requirements of *B. succinogenes*. The organism studied in the present work has properties which most closely ally it to the rumen *Bacteroides* spp. group, i.e. it is a Gram-negative, non-flagellated, asporogenous, curved rod with rounded ends. Although it cannot hydrolyse cellulose and is iodophilic, it does attack soluble pentosans (hemicelluloses), requires bicarbonate, factors in rumen liquor and a fermentable carbohydrate for *in vitro* proliferation. It produces end products typical of those found in normal rumen liquor from a variety of carbohydrates. Bryant & Burkey (1953) isolated many strains of bacteria from the bovine rumen which were similar to the 'less actively cellulolytic rod' of Hungate (1950). Many of their strains failed to attack cellulose even after prolonged incubation. Huhtanen & Gall (1953) also isolated Gram-negative curved rods similar to those of Hungate. It is interesting to note that some of Hungate's cellulolytic isolates were considered to migrate through agar in a manner similar to the cytophagas but he could not observe any creeping or bending movement. *B. amylogenes* is clearly flexible when examined in capillary tube preparations or when the phase microscope is used.

Despite similarities and differences of the rumen *Bacteroides* spp. group previously described and *B. amylogenes*, it is emphasized that the taxonomic assignment is purely provisional. It is possible that another, more suitable, place may be found. It might be contended that *B. amylogenes* be considered with the genus *Desulphovibrio*. Lewis (1954) found that sulphate reduction in the rumen occurs rapidly and suggested that species of this genus might be involved. However, absence of flagella and apparent failure to reduce inorganic sulphate to H_2S argue against this consideration. Great difficulty is encountered in attempting to classify bacteria from unique environments (as the rumen), using a system wherein generic descriptions are based mainly on medically important members. Yet, it is probably better to assign names to isolates, however tentative, rather than apply taxonomically meaningless letters or numbers to them.

The bicarbonate requirement of *Bacteroides amylogenes* is consistent with observations of Hungate (1950) and Bryant & Burkey (1953). Although the nutritional requirements of *B. amylogenes* were not studied systematically it is probable that some of the unknown factors supplied by rumen liquor are to be discovered among the constituents of medium 8. Yeast extract and proteose-peptone alone, or the completely defined medium alone, do not support good growth, whereas they do so when in combination. X agar, a medium in which

the only non-defined constituent is rumen liquor, likewise supports luxuriant growth. Obviously the factors required are in rumen liquor, an observation made by Hungate (1950) and Bryant & Doetsch (1954*a*). In general, it appears that commercially available peptones, usually adequate for cultivation of nutritionally fastidious bacteria, do not contain all the growth factors required for adequate *in vitro* growth of many authentic rumen bacteria. McNeill *et al.* (1954) came to this conclusion after a study of the nutritional requirement of the total rumen bacterial population. Wilson & Briggs (1955) have been unable to confirm this and found 'reinforced clostridial medium' of Hirsch & Grinsted (1954) a satisfactory replacement for rumen liquor.

The foregoing studies suggest that certain purely physiological criteria might be advanced for judging the authenticity of a bacterium isolated from the rumen. These would include: (1) optimum temperature about 39°; (2) ability to grow anaerobically and consequently a need for some reducing agent in media devised for *in vitro* cultivation; (3) requirement for some organic factor for *in vitro* growth, the factor to be found in rumen liquor and not necessarily in commercially available peptones; (4) production of fatty acids from amino acids and carbohydrates, these to be characteristic of those found in normal rumen liquor. Undoubtedly other conditions can be added as work progresses.

Bacteroides amylogenes survived 5 days in the presence of atmospheric O₂. It did not grow under these conditions, but did so promptly when subcultured and incubated anaerobically. There are reports in the literature stressing elaborate precautions taken to exclude the atmosphere from rumen liquor samples. It would appear that they are largely unnecessary. Oxygen may be inhibitory to authentic rumen bacteria; it is very doubtful whether it is lethal (Doetsch *et al.* 1952). The transfer of the rumen bacterial flora from mature animal to young must of necessity proceed through space containing oxygen, and it is likely that such organisms suspended in rumen liquor are adequately protected from any ill-effects of atmospheric O₂ by reducing substances contained therein.

Bacteroides amylogenes can transform into 'starch' a portion of any compound which serves as an energy source (Table 1). In this regard it is unlike iodophilic strains of *Streptococcus bovis* (Hobson & Mann, 1955) which produce iodophilic polysaccharide only from a very limited range of carbohydrates. Iodophilic polysaccharide also is produced from many carbohydrates by mixed suspensions of bovine rumen bacteria (Robinson *et al.* 1955). The transformation of energy sources to polysaccharide is an important but not exclusive characteristic of many authentic rumen bacteria. The reaction may represent a mechanism, developed during the evolution of the rumen bacterial flora, for dealing with an excess of soluble fodder carbohydrate.

The 'starch' is a reserve substance which is used during starvation periods. The utilization of this internal polysaccharide (probably via a phosphorylase, but not excluding a non-diffusible amylase) proceeds even in the presence of exogenous soluble carbohydrates. Either additional factors are required or conditions under which the experiments are performed prevent the utilization

of them. In any event, mixed suspensions of rumen bacteria readily utilize exogenous soluble carbohydrate and 'spare' their internal polysaccharide, or add to it (Gibbons *et al.* 1955) when continuous growth is not possible. *Bacteroides amylogenes*, in resting suspensions, does not produce polysaccharide even when its internal supply is depleted. Depletion requires 3–5 hr. at 39° in a resting suspension, a period of time which perhaps leaves the organisms moribund.

The reserve polysaccharide of *Bacteroides amylogenes* is typical of those so far described for authentic rumen organisms, i.e. it is composed solely of glucose units. It may be emphasized that all known intracellular, as distinct from capsular polysaccharides, are glucose polymers (Oxford, 1951; Gibbons *et al.* 1955; Hobson & Mann, 1955). The good yield of polysaccharide in this instance was due to the relatively large quantities of 'starch' stored as well as the ease with which the organisms can be ruptured.

In line with the above criteria for establishing the authenticity of a bacterium isolated from the rumen, it is of interest to note that the end products from D (+) xylose fermentation are typical of compounds found in rumen liquor. The negligible lactic acid production by *Bacteroides amylogenes* was not unexpected since other pentose-fermenting rumen bacteria do not produce this metabolite from pentoses (Heald, 1952; Doetsch *et al.* 1953). It should be borne in mind, that organisms other than the lactic acid bacteria may be responsible for the occasional traces of lactic acid found in normal rumen liquor, or the large amounts found in abnormal rumen liquor.

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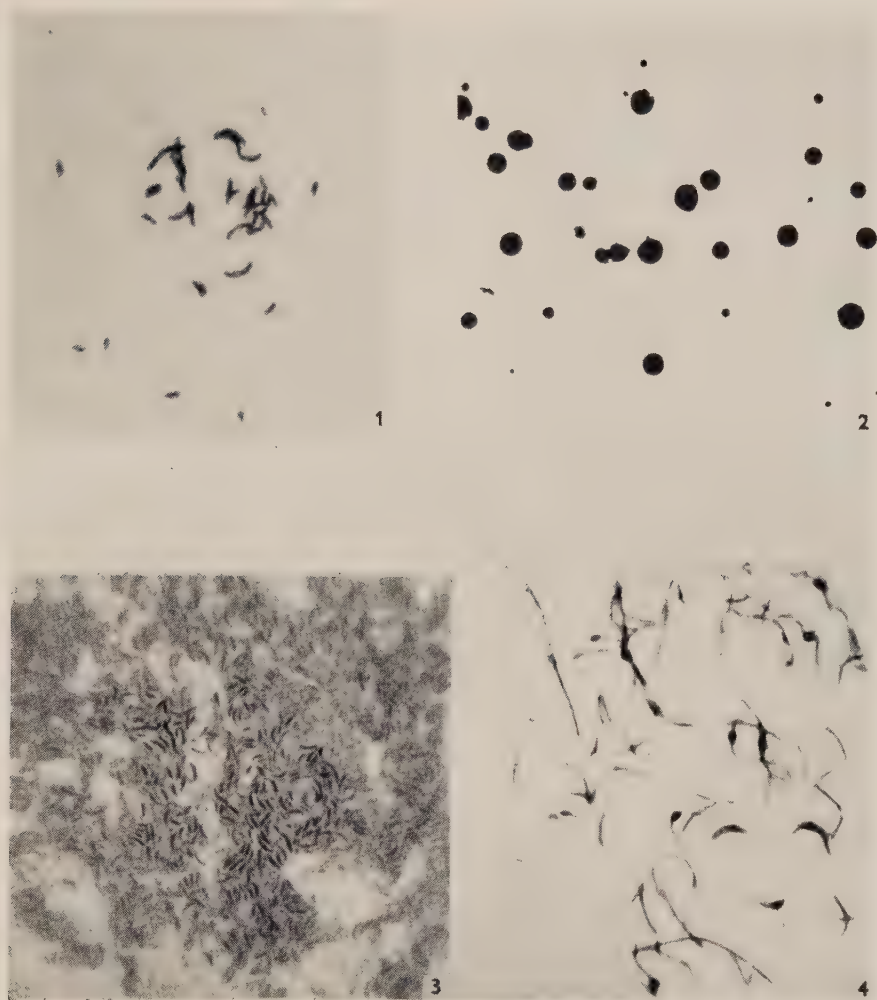
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EXPLANATION OF PLATE

- Fig. 1. Crystal violet stain of 20 hr. culture grown on X agar. $\times 880$.
- Fig. 2. Colonies of 20 hr. culture grown on X agar and covered with Lugol's iodine solution. $\times 2$.
- Fig. 3. Wet preparation of Lugol's iodine-treated cells taken from 20 hr. culture grown on X agar. $\times 880$.
- Fig. 4. Long forms and 'bulbs' produced in presence of toxic amounts of oxygen. $\times 850$.

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R. N. DOETSCH, B. H. HOWARD, S. O. MANN AND A. E. OXFORD—POLYSACCHARIDE
PRODUCTION BY A RUMEN BACTERIUM. PLATE 1

(Facing p. 168)

Adaptive Variation in the Level of Oxidative Activity in *Saccharomyces cerevisiae*

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SUMMARY: After growth in a medium containing 2 % (w/v) galactose, a strain of *Saccharomyces cerevisiae* showed greater oxidative activity, an essentially unchanged anaerobic fermentation rate and an increase in total growth, compared with yeast grown in the same medium but containing instead 2 % (w/v) glucose as major carbon source. The galactose-grown yeast possessed a generally higher level of oxidative metabolism, with enhanced $Q_{O_2}^{air}$ values (up to 500 %) with glucose and other oxidizable substrates, a higher cytochrome content and a greatly increased cytochrome oxidase activity. These differences were associated with an inhibitory effect of glucose on the formation of oxidative enzymes when this sugar was present in the growth medium at concentration greater than 0.05 % (w/v). Changes in the composition of the basal growth medium greatly altered the magnitude of the differences between the glucose-grown and galactose-grown organisms and the differences were not apparent with certain growth media. Possible interpretations of these findings are discussed.

The adaptation of yeast and other micro-organisms to the utilization of galactose or other saccharides has been widely employed as the test system in studies on the mechanisms of enzymic adaptation (Stephenson & Yudkin, 1936; Monod, 1942; Spiegelman, 1946). Such adaptations involve the formation of enzymes for the conversion of these sugars into intermediates of glucose or ribose metabolism; the existence of independent routes for metabolism of other sugars has not been clearly demonstrated. Thus, in the adaptive utilization of galactose in yeast a specific galactokinase is formed for conversion of galactose to galactose-1-phosphate (Trucco, Caputto, Leloir & Mittelman, 1948), and a second adaptive enzyme, galactowaldenase, converts galactose-1-phosphate to glucose-1-phosphate (Caputto, Leloir, Cardini & Paladini, 1950). The glucose-1-phosphate is assumed to be then metabolized by the usual fermentative and oxidative routes of glucose metabolism. Adapted yeast can utilize galactose either oxidatively or fermentatively (Sheffner & Lindegren, 1952). In growth experiments with *Streptococcus pyogenes*, White, Steele & Pierce (1955) observed a partial shift from homolactic metabolism with glucose as carbon source to a heterolactic metabolism with galactose. However, the possibility of the development of alternate pathways or of alterations in the balance of existing metabolic patterns as a result of adaptation appears not to have been thoroughly examined in yeasts.

It therefore seemed of interest to study further the observation made in this laboratory (Dr June Lascelles, personal communication) that after growth on galactose a strain of *Saccharomyces cerevisiae* oxidized glucose more rapidly

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than did the unadapted yeast grown on the same type of medium but utilizing glucose as major carbon source. The present work was an attempt to define this phenomenon more precisely. A preliminary report of this study has been presented previously (Strittmatter, 1955).

METHODS

Saccharomyces cerevisiae, strain no. 77 of the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey, England), was used in this work. The organism has been maintained in our laboratory on agar slopes, the medium containing inorganic salts, 2.25% (w/v) Difco malt extract, 0.05% (w/v) Difco yeast extract and 0.5% (w/v) sucrose.

Preparation of yeast suspensions

An 18 hr. agar slope culture of the organism on the maintenance medium was harvested by centrifugation, washed once with water and resuspended in water to yield a suspension containing the equivalent of 0.3 mg. dry wt. organisms/ml. Samples (0.3 ml.) of this suspension were added to 50 ml. samples of liquid growth medium in vessels shaped like an inverted T, similar to those used by Monod, Cohen-Bazire & Cohn (1951) except that the horizontal limbs of the vessels had a volume of 140 ml. Except where otherwise specified, the liquid growth medium contained 17.5% (v/v) of a 10% (w/v) tryptic digest of casein prepared by the method of Cole & Onslow (1916), 0.05% (w/v) Marmite (a commercial autolysate of yeast), and either 2% (w/v) glucose or 2% (w/v) galactose; all growth media were initially adjusted to pH 5.3. The vessels were incubated at 30° and were shaken at 36 oscillations/min. to ensure aeration of the medium. Unless otherwise indicated, the organisms were harvested by centrifugation at equivalent physiological ages, 1 hr. before the end of the period of logarithmic growth; this time of harvest ranged from 18 to 40 hr., depending upon the medium employed. The organisms were washed three times with cold distilled water (10 ml./50 ml. culture medium) and were resuspended in distilled water immediately before use, unless otherwise indicated. The washed organisms were generally used at once, but might be stored at 2° for at least 2 days without appreciable change in activity. Culture densities and the dry weight of harvested suspensions were estimated with an EEL photoelectric colorimeter (Evans Electroselenium Ltd.) or a Spekker Absorptiometer (Hilger and Watts Ltd.) which were calibrated for the organism.

Preparation of organisms for assay of cytochrome oxidase

Heated suspensions (Keilin, 1929). A 2% (w/v) suspension of organisms in 0.05 M-phosphate (pH 7.1) was warmed quickly to 50° in a water bath, kept at that temperature for 90 min., cooled at 25° and diluted appropriately for assay.

Cell-free extracts. A 50% (w/v) suspension (4 ml.) of the yeast in 0.1 M-phosphate (pH 7.1) was mixed with 10 g. glass balls (Ballotini no. 13) and shaken in

the Mickle disintegrator (Mickle, 1948) at 2° until microscopic examination indicated that at least 90 % of the organisms were disrupted. The glass balls, unbroken organisms and cellular debris were removed by centrifugation in the cold, and the opalescent, cell-free supernatant solution used as enzyme preparation.

Manometric determinations

Rates of oxygen uptake in air ($Q_{O_2}^{air}$) and of carbon dioxide evolution in nitrogen ($Q_{CO_2}^{N_2}$) were determined by conventional techniques in the Warburg respirometer at 30°. Suitable amounts of the yeast preparations, generally 2–10 mg. dry wt., were suspended in 0.033 M-phosphate buffer (pH 6) containing any additional substances as noted in the text. Substrates were added from the side-arm after equilibration to give a final concentration of either 0.01 M (for hexoses) or 0.02 M (for other substrates). In oxygen uptake measurements, 20 % (w/v) KOH was placed in the centre well; for measurements on cyanide inhibition of respiration, this was replaced with appropriate KOH-KCN mixtures (Umbreit, Burris & Stauffer, 1949).

For cytochrome oxidase assays on heated suspensions, the substrate was 0.02 M-*p*-phenylenediamine and the vessels contained 0.033 M-phosphate buffer (pH 7.1); in the assays on cell-free extracts, the substrate was 0.02 M-ascorbate and the vessels contained 0.033 M-phosphate buffer (pH 7.1), 0.01 M-NaCl and 3×10^{-5} M-cytochrome *c*. The recorded values have been corrected for autoxidation of the substrates; in the assays with ascorbate the autoxidation rate was measured by extrapolating to an enzyme concentration of zero from a series of different enzyme concentrations (see Umbreit *et al.* 1949).

The $Q_{O_2}^{air}$ and $Q_{CO_2}^{N_2}$ values were calculated for a period of linear activity, generally for the first hour, and are expressed as μ l. gas/mg. dry wt. yeast/hr. Except where otherwise specified, all $Q_{O_2}^{air}$ and $Q_{CO_2}^{N_2}$ values have been corrected for endogenous values; the trends of the results are not altered by omitting these corrections but the corrected values are felt to be a better expression of the activities studied. The endogenous values did not vary appreciably in the different preparations and, with exceptions indicated in the text, were only a small percentage of the values obtained with added substrate. The endogenous $Q_{O_2}^{air}$ value was 2–3.5 for organisms grown on the standard glucose-containing media and 3–5 for those grown on galactose-containing media, while the endogenous $Q_{CO_2}^{N_2}$ value was 0–2 and may be considered negligible.

Determination of cytochrome c

Quantitative estimation of cytochrome *c* content in yeast suspensions was made with a microspectroscope assembly (kindly made available by Dr W. E. van Heyningen). A solution of commercial cytochrome *c* was used as a standard, and sodium hydrosulphite (dithionite) was added to both suspensions and standard to maintain the cytochrome in the reduced state.

RESULTS

Characteristics of organisms grown in the standard glucose and galactose media

Growth. The nature of the sugar added to the basal casein digest + Marmite growth medium influenced both the kinetics of growth and the maximum yields of organisms obtained (Fig. 1). In the presence of glucose, growth began without appreciable delay and the final yield was equivalent to 1.1 mg. dry wt. organisms/ml. medium. With galactose there was a lag period of about 6 hr.,

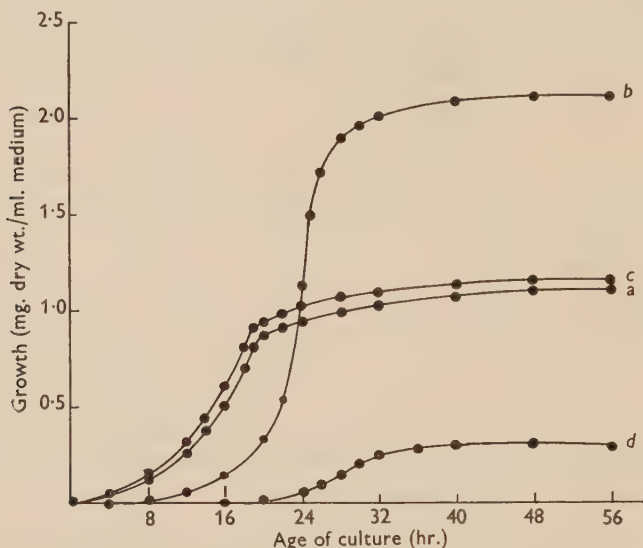


Fig. 1. Effect of sugar in growth medium on growth of *Saccharomyces cerevisiae*. Unadapted yeast was grown in the basal casein digest + Marmite medium to which was added: (a) 2% (w/v) glucose; (b) 2% (w/v) galactose; (c) 2% (w/v) glucose + 2% (w/v) galactose; (d) no sugar added.

reflecting the need for this organism to adapt to utilization of galactose, but the final growth obtained was twice as great as that obtained with the glucose medium. Mixtures of glucose and galactose produced growth curves approximating that obtained with glucose alone, while omission of any added sugar resulted in only poor growth after a long and variable lag period of 18–24 hr.

Oxidation and fermentation of glucose. Organisms growing in the standard casein digest + Marmite medium containing either glucose or galactose were harvested at various ages and their ability to oxidize and ferment glucose was determined. For any given growth time, galactose-grown organisms oxidized glucose much more rapidly than did glucose-grown organisms (Fig. 2a); this difference was of the same magnitude whether or not a correction for endogenous oxygen uptake was made. Except for organisms from very young cultures (12 hr.), galactose-grown organisms also fermented glucose at a greater rate than did the glucose-grown ones (Fig. 2b).

Since both oxidative and fermentative capacities decreased with age in both

types of yeast, further experiments in this study were carried out with organisms of the same physiological age, harvested 1 hr. before the end of the logarithmic phase of growth. As can be seen from Fig. 1, this age for harvesting was at about 18 hr. for glucose-grown organisms and at about 24 hr. for galactose-grown organisms. When compared at these equivalent physiological ages, the galactose-grown organisms oxidized glucose 5.7 times as rapidly as the glucose-grown ones (Fig. 2a); in a series of ten preparations, the average (corrected) values of $Q_{O_2}^{air}$ (glucose) were $50.7 (\pm 2.8)$ and $8.8 (\pm 0.9)$, respectively. The $Q_{CO_2}^{Na}$ (glucose) level was about 150 for both types of organism (Fig. 2b).

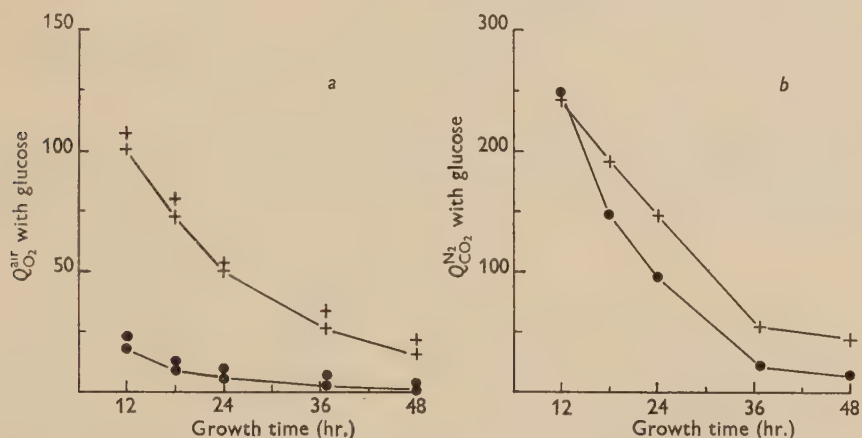


Fig. 2a, b. Effect of age of culture on ability of harvested *Saccharomyces cerevisiae* to (a) oxidize glucose, and (b) ferment glucose anaerobically. Organisms were harvested at indicated times from standard casein digest + Marmite medium containing either 2% (w/v) glucose (●—●) or 2% (w/v) galactose (+—+) and were assayed manometrically with 0.01 M-glucose as substrate. In Fig. 2a, the lower of each pair of points represents the activity corrected for endogenous respiration; the upper point is uncorrected.

Oxidation and fermentation of galactose. The unadapted glucose-grown yeast did not metabolize galactose appreciably, while the galactose-grown organisms both oxidized and fermented this sugar (Table 1). It is of interest that the

Table 1. *Metabolism of glucose and galactose by Saccharomyces cerevisiae grown in media containing either glucose or galactose*

Assay	Growth conditions		
	Glucose (18 hr.)	Galactose (24 hr.)	Galactose (44 hr.)
$Q_{O_2}^{air}$ (glucose)	9.5	52.4	17.6
$Q_{O_2}^{air}$ (galactose)	0	15.8	17.9
$Q_{CO_2}^{Na}$ (glucose)	148.0	151.0	52.0
$Q_{CO_2}^{Na}$ (galactose)	1.0	138.0	49.5

Organisms were harvested at the times indicated and assayed manometrically with 0.01 M-glucose or galactose as substrate.

24 hr. galactose-grown organisms oxidized galactose at only one-third the rate observed with glucose although both sugars were fermented at similar rates; in older cultures the oxidative rates for the two sugars approached equality. Since, however, the rates of both oxidation and fermentation of glucose were decreasing markedly with age (Fig. 2), it is difficult to deduce evidence for changing metabolic patterns from such data.

Oxidation of other substrates. The high rate of glucose oxidation by the galactose-grown yeast reflected a generally higher level of oxidative activity in these organisms, compared with those grown on glucose. Of the substrates tested, all those attacked by the yeasts were oxidized more rapidly by the galactose-grown organisms (Table 2).

Table 2. *Respiration of glucose-grown and galactose-grown Saccharomyces cerevisiae in the presence of various substrates*

Harvested organisms were assayed by conventional manometric techniques at 30° in 0.033 M-phosphate, pH 6.0. Acid substrates were neutralized with sodium hydroxide to pH 6.0 before addition. The endogenous respiratory rate was not subtracted in calculating the $Q_{O_2}^{\text{air}}$ values in this table.

Substrate	$Q_{O_2}^{\text{air}}$	
	Glucose-grown yeast	Galactose-grown yeast
None	3.0	4.5
Glucose, 0.01 M	13.2	54.7
Ethanol, 0.02 M	13.3	52.1
Lactate, 0.02 M	5.4	31.9
Pyruvate, 0.02 M	3.6	19.2
Acetate, 0.02 M	2.6	4.5
Succinate, 0.02 M	3.0	6.0
Fructose diphosphate, 0.01 M	2.6	4.5

There is no evidence that these parallel differences between the two kinds of organism derived from a generally greater permeability of the galactose-grown ones to the substrates. Glucose freely entered both types, as indicated by the same high rates of anaerobic fermentation in both (Fig. 2*b*). On the other hand, succinate and fructose diphosphate, the two test substances which are known to be seriously affected by a permeability barrier at the pH value of the assay system, were not rapidly attacked by either type. Of the other substrates, pyruvate is known to permeate the yeast cell wall, though less readily than at an acid pH value, and the remaining substances pass freely (see Barron, Ardao & Hearon, 1951; Eaton & Klein, 1954).

Electron transport system. The generally higher level of oxidative activity in the galactose-grown organisms suggested a greater capacity of its terminal respiratory system for transport of electrons to oxygen. If grown aerobically, most strains of *Saccharomyces cerevisiae* develop the cyanide-sensitive cytochrome-cytochrome oxidase system as the major pathway of electron transport (Warburg, 1927; Keilin, 1929). This system also predominated in the yeasts used in the present study, since cyanide (10^{-3} M) produced 85–90 % inhibition of glucose oxidation and about 95 % inhibition of lactate oxidation

by suspensions of both glucose-grown and galactose-grown organisms. The concentration of components of the cytochrome-cytochrome oxidase system in the two kinds of yeast was therefore compared (Table 3).

Table 3. *Cytochrome oxidase activity and cytochrome c content of glucose-grown and galactose-grown Saccharomyces cerevisiae*

Preparation used	Assay	Activity	
		Glucose-grown yeast	Galactose-grown yeast
Heated yeast suspension	$Q_{O_2}^{air*}$ (endogenous)	0.1	0.4
	$Q_{O_2}^{air*}$ (<i>p</i> -phenylenediamine)	0.9	5.7
Cell-free extract	$Q_{O_2}^{air*}$ (endogenous)	0	0
	$Q_{O_2}^{air*}$ (ascorbate)	0.9	9.1
	$Q_{O_2}^{air†}$ (ascorbate)	3.0	26.3
Untreated yeast suspension	Cytochrome <i>c</i> . (μ mole/mg. dry wt.)	0.64×10^{-4}	1.08×10^{-4}

The $Q_{O_2}^{air}$ values are corrected for autoxidation of substrate but not for endogenous respiration.

* μ l. O_2 /mg. dry wt. of original yeast suspension/hr.

† μ l. O_2 /mg. dry wt. of cell-free extract/hr.

Cytochrome oxidase activity was determined manometrically both on heated suspensions with *p*-phenylenediamine as substrate and on cell-free extracts with ascorbate as substrate, as described in Methods. The ratio of activities for galactose-grown against glucose-grown organisms was about 6.5:1 by assay of heated organisms and 10:1 by assay of the cell-free extracts. The activities of the cell-free extracts were calculated both on the basis of dry weight of the original suspensions and on the basis of 'dry weight' of the extracts, determined by drying the material precipitable from the extracts by trichloroacetic acid. The ratio of activities was nearly the same by both methods of calculation, indicating that both cell-free extracts represented approximately the same proportion (about one-third) of the original whole cell substance.

The $Q_{O_2}^{air}$ values obtained by either assay fall below the minimum physiological capacity of the cytochrome oxidase system (presumably represented by the $Q_{O_2}^{air}$ values obtained with glucose or ethanol as substrate). These low activities probably reflect non-optimal conditions of the assay procedures and loss of enzyme activity during preparation of extracts or heated suspensions. However, the fair agreement of the activity ratios obtained by independent assay systems supports the conclusions that the cytochrome oxidase activity of galactose-grown organisms is markedly greater than that of glucose-grown ones.

The cytochrome *c* content, as determined on suspensions, was only 70 % higher in galactose-grown organisms. This small increase in cytochrome *c*, in contrast to the great increase in cytochrome oxidase activity, may be compared with observations of Slonimski (1955) on anaerobically-grown yeast: exposure to oxygen induced a 200-fold increase in cytochrome oxidase activity, but only a 20-fold increase in cytochrome *c* content.

Adaptive (inductive) nature of the phenomenon

Two types of experiments were employed to test whether the differences in oxidative capacities between glucose-grown and galactose-grown organisms result from adaptive (inductive) changes or from selection of different strains (or mutants) in the growth media.

First, following seven subcultivations in the standard galactose-containing liquid medium, the harvested organisms had a $Q_{O_2}^{\text{air}}$ (glucose) value of 52, typical of galactose-grown organisms. If after such repeated galactose cultivation the yeast was grown once in the standard glucose medium, the $Q_{O_2}^{\text{air}}$ (glucose) value of harvested organisms was 9.

Secondly, an inoculum from the stock yeast culture was streaked on agar plates of the same composition as the maintenance medium but with 2% (w/v) galactose replacing 0.5% (w/v) sucrose as chief carbon source. After growth a single colony was chosen to streak a new plate, and after ten such transfers a single colony was used as inoculum for growth in casein digest + Marmite liquid medium containing 2% (w/v) glucose or 2% (w/v) galactose. The organisms were harvested and assayed manometrically. In three independent experiments, the organisms thus grown in galactose medium possessed an average $Q_{O_2}^{\text{air}}$ (glucose) value of 53.2 (51.2, 53.6, 54.9); the glucose-grown organisms possessed a $Q_{O_2}^{\text{air}}$ (glucose) value of 9.0 (8.6, 8.9, 9.5).

In both types of experiments, therefore, repeated exposure to conditions favouring selection of highly oxidative strains did not alter the oxidative capacity of the galactose-grown yeast, while a single passage through glucose-containing medium reduced the oxidative level to that normally observed with glucose-grown organisms. The results strongly suggest that the differences in oxidative levels between these glucose-grown and galactose-grown yeasts are adaptive (inductive) in origin.

Factors which affect the differences in growth and oxidative capacity

Strain of yeast. The differences between glucose-grown and galactose-grown yeast with respect to growth and oxidative capacity were not unique to *Saccharomyces cerevisiae* strain no. 77, but were also observed with the 'Yeast Foam' strain of *S. cerevisiae* (kindly supplied by Dr P. Slonimski). However, the differences were not as great with the 'Yeast Foam' strain.

Concentration of sugar in the growth medium. The oxidative activity of harvested organisms was markedly decreased when the initial concentration of glucose in the growth medium exceeded a very low optimal concentration, while high initial concentrations of galactose caused a relatively slight decrease of oxidative activity (Table 4). The anaerobic fermentation rates were not appreciably altered by these manipulations. With the lowest concentration of glucose tested, 0.5 mg./ml. growth medium, the organisms possessed a $Q_{O_2}^{\text{air}}$ (glucose) value approaching that found with galactose-grown organisms, while with concentrations of 10 mg./ml. or more, the oxidative rate was less than 20% of that of galactose-grown organisms. With galactose, increasing the concentration from 1 to 200 mg./ml. decreased the $Q_{O_2}^{\text{air}}$ (glucose) value of the

resultant organisms by only about 20 %. The sugar concentrations required to produce maximum oxidative activities may be lower than the minimum concentrations employed. However, this possibility could not be tested since appreciably lower concentrations had the same effect as entirely omitting

Table 4. *Effect of varying the sugar concentration of the growth medium on metabolic activity of Saccharomyces cerevisiae*

Glucose or galactose was added to the basal casein digest + Marmite medium in the concentrations indicated. Organisms were grown and harvested in the standard manner and assayed manometrically with 0.01 M-glucose as substrate.

Initial concentration of sugar in growth medium (mg./ml.)	Activity			
	Glucose-grown yeast		Galactose-grown yeast	
	$Q_{O_2}^{air}$	$Q_{CO_2}^{N_2}$	$Q_{O_2}^{air}$	$Q_{CO_2}^{N_2}$
0.5	52.0	163	—	—
1.0	39.5	174	60.9	180
2.0	30.1	160	59.8	162
10.0	10.5	153	—	—
20.0	8.8	148	51.3	156
50.0	7.9	150	49.7	160
200.0	—	—	50.4	163

added sugar from the basal growth medium: there were long lag periods and poor growth (Fig. 1), and the organisms obtained had low metabolic activities (Table 5).

Table 5. *Effect of varying the type of sugar in the growth medium on growth and metabolic activity of Saccharomyces cerevisiae*

Sugars as indicated were added to the basal casein digest + Marmite growth medium; growth, harvesting and assays were carried out by the standard procedures. The $Q_{O_2}^{air}$ and $Q_{CO_2}^{N_2}$ values were obtained with the harvested organisms using 0.01 M-glucose as substrate. Growth values represent the levels reached if the cultures were permitted to grow to maximum density. Values given are the average of results from four to ten experiments.

Sugar added to growth medium	Activity of harvested organisms		Maximum growth (mg./ml.)	Increase in lag phase* (hr.)
	$Q_{O_2}^{air}$	$Q_{CO_2}^{N_2}$		
2 % (w/v) glucose	8.8	148	1.10	(0)
2 % (w/v) galactose	50.7	153	2.20	6
2 % (w/v) glucose + 2 % (w/v) galactose	14.3	135	1.15	0
2 % (w/v) fructose	9.0	166	1.18	0
2 % (w/v) mannose	15.0	161	1.25	2
None added	< 5	< 25	0.30	18–24

* Increase in duration of lag phase over that obtained with glucose.

These results suggest that the relatively low oxidative activity of the glucose-grown organisms derives primarily from an inhibitory effect of glucose on the development of oxidative capacity in the growing organisms, i.e. on the formation of the enzymes involved in oxidative metabolism. However, the

possibility of an additional stimulatory effect of galactose is not thereby ruled out.

Type of sugar in growth medium. Exploratory experiments showed that the glucose-grown yeast could, without preliminary adaptation, ferment or oxidize fructose and mannose approximately as fast as it did glucose. Yeast hexokinase is known to phosphorylate fructose and mannose readily (Berger, Slein, Colowick & Cori, 1946) and the phosphorylated derivatives enter the main stream of glucose metabolism. It is therefore significant that when used as growth substrates (Table 5) these sugars produced organisms similar in their low oxidative activities to those grown in glucose medium; in addition, the growth curve and yield of organism with these sugars were similar to those obtained with glucose.

Organisms grown in a mixture of glucose and galactose also were similar in their growth characteristics and low oxidative activity to those grown in glucose alone (Table 5). Apparently, these organisms produced preferentially the balance of enzyme constituents required for the less oxidative metabolic pattern evoked by high concentrations of the normal 'constitutive' substrate, glucose. These glucose + galactose-grown organisms were partially adapted to utilization of galactose at the standard age of harvest (18 hr.): the $Q_{O_2}^{air}$ and $Q_{CO_2}^{N_2}$ values with galactose as substrate were 5.4 and 7.0, respectively. Adaptation increased with age of culture, e.g. at 24 hr. $Q_{CO_2}^{N_2}$ (galactose) was 27, but the decrease of metabolism activities with age of culture became the more significant factor, e.g. at 24 hr. the $Q_{O_2}^{air}$ (galactose) and $Q_{O_2}^{air}$ (glucose) values, while now nearly equal, had dropped to 4.2 and 5.3, respectively.

Changes in medium during growth. Since the acidity of the environment may determine what type of metabolism develops in a micro-organism (White *et al.* 1955), the pH value of the glucose and galactose growth media was determined at various ages of culture. The acidity increased in both media in proportion to the amount of growth. At the standard times of harvest the pH value of the glucose medium was 4.5 and that of the galactose medium, 4.2. This parallel drop of pH value and lack of gross difference in acidity between the two cultures appears to rule out acidity as a significant factor in the different metabolic patterns of glucose-grown and galactose-grown yeasts.

The decline in the concentration of added sugar in the medium with time was also examined, since exhaustion of hexose might stimulate increased development of oxidative metabolism (Swanson & Clifton, 1948). The remaining sugar of the growth media was separated by paper chromatography, eluted and assayed by an anthrone method (Koehler, 1952). More than one-fourth of the glucose or galactose initially present remained at the standard ages of harvest, hence the increased oxidative activity of the galactose-grown organisms cannot be attributed to an early exhaustion of galactose from the growth medium.

Composition of the basal growth medium. The difference between the glucose-grown and galactose-grown yeasts with respect to oxidative activity and maximum growth were profoundly affected by altering the composition of the basal growth medium (Table 6). For organisms of equivalent physiological ages the

oxidative activity was similar in galactose-grown organisms harvested from different media (column 6), while the oxidative activity of glucose-grown organisms was greatly altered by change of growth medium (column 3). Variation in the ratio of oxidative activities in the two types of yeast therefore largely reflects the effect of the medium on the inhibition by glucose of oxida-

Table 6. *Influence of composition of growth medium on growth and metabolic activity of Saccharomyces cerevisiae*

Organisms were grown in the various basal media to which were added either 2% (w/v) glucose or 2% (w/v) galactose; all media were initially adjusted to pH 5.3. The $Q_{O_2}^{air}$ and $Q_{CO_2}^{N_2}$ values with glucose as substrate were obtained with organisms harvested at equivalent physiological ages late in the period of logarithmic growth. Growth values represent the levels obtained when the cultures were permitted to grow to maximum density. Values given in this table represent the average of results from three to ten experiments.

Medium no.	Composition of basal medium (2)	Glucose-grown yeast			Galactose-grown yeast		
		$Q_{O_2}^{air}$	$Q_{CO_2}^{N_2}$	Yield (mg./ml.)	$Q_{O_2}^{air}$	$Q_{CO_2}^{N_2}$	Yield (mg./ml.)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	Casein digest + 0.05% (w/v) Marmite	8.8	148	1.10	50.7	153	2.20
2	Casein digest + 0.025% (w/v) Marmite	6.5	166	0.38	43.7	163	0.78
3	Casein digest + 0.2% (w/v) Marmite	15.8	174*	1.93	56.8	164*	3.44
4	Casein digest + 0.5% (w/v) Marmite	45.6	250*	5.28	73.7	175*	5.32
5	0.05% (w/v) Marmite + 0.1 M-phosphate	36.3	165	0.58	42.7	157	0.65
6	0.4% (w/v) NH_4Cl + 0.05% (w/v) Marmite + 0.1 M-phosphate	28.2	183	0.65	67.3	191	0.81
7	Chemically defined	22.4	215*	1.99	55.4	243*	1.79
8	Yeast extract-malt extract-salts	72.9	195	11.0	74.7	163	11.5

* Not corrected for endogenous activity.

tive enzyme formation. The maximum growth for both glucose-grown and galactose-grown organisms varied with the different media (columns 5 and 8, respectively), but variation in the relative growth of the two types again reflects the influence of the medium on the inhibitory effect of glucose. The anaerobic fermentation rate was of the same order of magnitude in both types for all growth media tested (columns 4 and 6, respectively). This constancy of fermentative capacity tends to confirm that the various yeast preparations were of comparable physiological ages.

Of various basal media examined, the most marked differences between glucose-grown and galactose-grown organisms were observed with the standard casein digest + Marmite medium; this medium was consequently used as a basis for the experiments reported in this paper. The results of varying the

concentration of Marmite (media 2, 3, 4) from that in the standard basal medium (medium 1) indicate that an increased Marmite concentration reduces the differences in oxidative activity. Since the Marmite concentration was the limiting factor in the amount of growth obtained, attempts to increase the inhibitory effect of glucose by drastic decrease of Marmite concentration proved impractical.

Omission of casein digest (medium 5) from the standard basal growth medium also resulted in glucose-grown organisms with maximum growth and oxidative activity approaching the levels found in galactose-grown ones. These results suggest that the presence of casein digest permits or potentiates the appearance of the differences between the two types of organism. The phosphate was added to medium 5 to provide buffering capacity; it had no specific effect, as indicated by the fact that addition of 0.1 M-phosphate to the standard growth medium (results not tabulated) produced no significant alterations in metabolic activities or growth.

To test further the effects of varying the major nitrogenous constituents, casein digest was replaced by ammonium chloride (medium 6) or ammonium sulphate was used as sole major nitrogen source (medium 7). In the latter case, the chemically defined medium contained, per litre, 2 g. $(\text{NH}_4)_2\text{SO}_4$, 7.5 g. KH_2PO_4 , 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 g. CaCl_2 , 2.8 mg. FeSO_4 , 0.4 mg. thiamine, 5 mg. inositol, 4 mg. biotin, 0.4 mg. pantothenate and 0.14 mg. *p*-aminobenzoic acid. With either medium, a partial decrease of the glucose inhibition of oxidative activity was obtained, while the maximum growth obtained was nearly the same for glucose-grown and galactose-grown organisms.

The fact that the differences between glucose-grown and galactose-grown organisms were least evident when Marmite was the major nitrogenous component of the growth medium suggests that a balance of substances in the growth medium which resembles the composition of the yeast aids in suppressing the inhibitory effects of glucose.

For comparison, the yeast was also grown in a complex medium (medium 8) identical with that used for the maintenance-agar slopes except for omission of agar and substitution of 2% (w/v) glucose or 2% (w/v) galactose for 0.5% (w/v) sucrose. This rich medium, on which the organisms had been maintained for a long period, produced high yields of yeast and high oxidative activity, with no appreciable differences between glucose-grown and galactose-grown organisms in these respects.

DISCUSSION

The ultimate mechanism by which glucose inhibits the development of oxidative capacity with this *Saccharomyces cerevisiae* strain is not certain, but several possible factors suggest themselves. First, the results obtained by varying the nature of the sugar present in the growth medium (Table 5) indicate that oxidative pathways are relatively little developed in the presence of high concentrations of a sugar readily and immediately fermentable without need for preliminary adaptation. Slonimski (1955) found that high concentrations of

glucose result in a decreased rate of oxygen-induced formation of certain oxidative enzymes in non-proliferating yeast suspensions; this retardation was associated with a heightened aerobic fermentation rate. Glucose is known to inhibit the formation of a number of enzymes in various organisms (Epps & Gale, 1942). In at least some instances, this glucose effect may be interpreted as a homeostatic mechanism whereby products formed by metabolism of glucose by 'constitutive' enzyme systems can inhibit the formation of 'adaptive' (inducible) enzymes whose activity would merely increase the metabolic pool of these products (Neidhardt, 1956). Glucose inhibition of oxidative enzyme formation in yeast may similarly reflect an intracellular regulatory mechanism; the 'constitutive' enzyme systems readily fill the energy requirements of the organism by fermentative breakdown of glucose when this sugar is present, and the presence of this metabolic energy pool may partially inhibit the formation of 'adaptive' energy-yielding oxidative enzyme systems.

Secondly, the results obtained by varying the composition of the growth medium (Table 6) suggest that glucose inhibition may involve depletion or imbalance of essential constituents required for formation of oxidative enzymes. Such deficiencies may result from increased utilization of essential components for other purposes or in other reactions not as prominent in a more oxidative metabolic milieu. The synthesis of enzymic protein requires the presence of a complete array of the component amino acids in sufficient quantity (Spiegelman & Halvorson, 1953), and for optimal utilization by the organism the various amino acids must be presented in correct proportions (Almquist, 1953). Various other substances, including nucleic acids, are also essential components of the protein synthesis mechanism (Gale & Folkes, 1955).

The reasons for the differences between glucose-grown and galactose-grown organisms reported here are obscure. Gross differences of pH value in the media during growth or an early depletion of galactose appear to be ruled out as significant factors in causing these differences. Further, organisms grown in glucose + galactose mixtures possess low oxidative activity and give a yield of organisms characteristic of glucose-grown ones (Table 5), despite their ability to metabolize galactose. It therefore seems improbable that galactose or some metabolic derivative of galactose required for essential structural or functional roles exerts a specific stimulatory effect on development of oxidative enzyme activity. The apparent stimulatory effect of galactose appears rather to arise from a relative lack of the glucose type of inhibition. The organism studied must adapt to utilization of galactose and then actively maintain the adaptive enzyme system; the consequent lower facility and capacity for fermenting this substrate, particularly in newly adapting cultures, may permit development of efficient oxidative pathways for more complete utilization of the available supply of substrates for energy production. Galactose may also evoke development of an alternate oxidative pathway perhaps characteristic of galactose metabolism but also utilizable by glucose in the adapted yeast; however, no direct evidence for such a pathway has been obtained.

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Autolytic Release and Osmotic Properties of 'Protoplasts' from *Staphylococcus aureus*

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SUMMARY: The cell wall of exponential phase *Staphylococcus aureus* (strain Duncan) loses its tensile strength in c. 2 hr. when the organisms are incubated at 25° in 1.2 M-sucrose at pH 5.8 and ionic strength 0.3. The 'protoplasts' thus released from the mechanical protection of the cell wall are stable in 1.2 M-sucrose but lyse in media of lower osmotic pressure. The mean internal osmotic pressure of the 'protoplasts' is c. 20 atmospheres; they are permeable to glycerol but not to sucrose or NaCl. The rate of 'protoplast' release varies with the rate of growth of the organisms at harvesting. After osmotic explosion of 'protoplasts' released from slowly growing organisms the plasma membranes may be recovered as spherical shells which disintegrate and condense into small particles on washing, and 75 % of the weight of the cell walls may be recovered as hemispherical shells.

The aim of the work described in this paper was to develop a method for releasing the protoplasts of *Staphylococcus aureus* (strain Duncan) from the protection of the cell wall so as to facilitate the study of the structure and function of the plasma membrane (Mitchell & Moyle, 1956*a*). Since staphylococci had been reported to be somewhat sensitive to lysozyme under certain conditions (Webb, 1948; Kern, Kingkade, Kern & Behrens, 1951) we decided to investigate the possibility of releasing stable 'protoplasts' of staphylococci by modifications of the technique of Weibull (1953) similar to those which we developed for the release of the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* (Mitchell & Moyle, 1956*b*).

METHODS

Growth and preparation of organisms. Cultures were grown at 25° in a medium containing 3 % (w/v) tryptic digest of casein, 1 % glucose and 0.1 % Marmite, either in Roux bottles or using the rotated flask technique (Mitchell, 1949). The organisms were harvested by centrifugation, washed once with distilled water and suspended at a concentration corresponding to c. 100 mg. dry weight/ml. in distilled water.

Measurement of turbidity and dry weight. The extinction of 1 cm. depth of bacterial suspension was measured at a wavelength of 700 m μ ., using the Beckman model DU spectrophotometer. The suspensions were diluted to give readings of log₁₀ (I_0/I) between 0.1 and 0.2 so that, the extinction being proportional to dry weight in this range, the dry weight could be estimated by multiplying the extinction by a factor. The factors for the different organisms were determined by calibrating the extinction against the dry-weight concentration estimated by drying c. 100 mg. samples to constant weight at 105°.

Light microscopy. The intact organisms, 'protoplasts' and membranes were examined with a Zeiss binocular phase-contrast microscope in films of aqueous solution sealed between slide and coverslip with vaseline.

Electron microscopy. The cell walls and plasma membranes were washed with large volumes of dust-free distilled water. Drops of dilute suspension in distilled water were placed on collodion film-covered specimen grids. The greater part of the drops was removed by touching them momentarily with a piece of clean filter-paper, and the remainder of the material was rapidly dried on the grids *in vacuo* over P_2O_5 . The specimens thus prepared were shadowed at an angle of 15° to the surface of the collodion film with gold-palladium and examined and photographed with the Siemens Elmiskop 1 electron microscope operated by Dr K. Deutsch.

Index of osmotic fragility. Samples (0.1 ml.) of the suspensions of organisms undergoing 'protoplast' release were pipetted into paired 5 ml. samples of 1.2 M-NaCl in 0.01 M-sodium phosphate and 0.01 M-sodium phosphate buffer alone at pH 6.8. The turbidities of the suspensions in the paired tubes were measured after 20 min. at 20° . It was often convenient to express these turbidities as percentages of the values obtained at the beginning of incubation in the 'protoplast'-releasing solutions when all the organisms were still osmotically insensitive, and to write an index of osmotic fragility a/b , a being the percentage of the initial turbidity in the buffer alone and b being that in the buffered saline. If the 'protoplasts' of the staphylococci studied here behaved like the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* (Mitchell & Moyle, 1956*b*), they would lyse in buffer alone but not in buffered 1.2 M-NaCl, and the turbidity of the lysed 'protoplasts' would be very small compared with that of the intact 'protoplasts'. The ratio a/b would therefore fall from unity towards zero as the organisms became osmotically fragile and the proportion of osmotically fragile organisms would be approximately $1 - (a/b)$.

RESULTS

Seven strains of *Staphylococcus aureus* (strains Duncan, Pusey, 9104 and 9963) and *S. albus* (strains 7834, 9958 and 9966) were grown in Roux bottles and harvested during the phase of decelerated growth. The organisms were suspended at a final concentration corresponding to *c.* 5 mg. dry weight/ml. with 0.5 mg. crystalline egg white lysozyme/ml. in 1.2 M-sucrose solutions in either 0.02 M-sodium phosphate buffer at pH 6.8 or in 0.02 M-tris-(hydroxymethyl)-aminomethane acetate buffer at pH 8.4 at 25° . Similar suspensions in which only the lysozyme was omitted were used as controls. The apparent rate of 'protoplast' release is shown by the index a/b (as defined above) in Table 1. Incubation in the presence of lysozyme at pH 6.8 made all the organisms appreciably more fragile to suspension in 0.01 M-phosphate buffer than to suspension in buffered 1.2 M-NaCl (a less than b), and the same was true for most of the organisms incubated at pH 8.4, although at this pH value the fragility of the organisms either before or after pipetting into the buffered NaCl was generally greater than at pH 6.8.

In one case (7834) the fragility in buffered NaCl was greater than in buffer alone. Even in the absence of lysozyme, however, there was a significant increase in the osmotic fragility of all the organisms incubated at pH 6·8 and in some of them incubated at pH 8·4. This experiment suggested that by appropriately adjusting the conditions it might be possible to release the protoplasts of staphylococci from the cell wall without the addition of lysozyme. Clearly, it would be advantageous if it were possible to eliminate the necessity

Table 1. *Development of osmotic fragility in seven strains of staphylococcus*

The organisms were incubated at a concentration of 5 mg./ml. at 25° in 1·2 M-sucrose buffered with 0·02 M-acetate (pH 6·8) or 0·02 M-tris (pH 8·4) without (–) or with (+) 0·5 mg. egg-white lysozyme/ml. Ratios *a/b* in the body of the table represent percentage initial turbidity on pipetting 0·1 ml. samples of cell suspension into 5 ml. 0·01 M-sodium phosphate buffer at pH 6·8 (*a*) without and (*b*) containing 1·2 M-NaCl. The values of *a/b* at zero time are, of course, 100/100.

		Strain of staphylococcus													
		Duncan		Pusey		9104		9963		7834		9958		9966	
		Presence (+) or absence (–) of lysozyme													
		–	+	–	+	–	+	–	+	–	+	–	+	–	+
Time		Percentage initial turbidity													
(hr.)	pH														
20	6·8	98/99	41/74	94/97	66/71	83/93	27/66	96/99	66/78	89/91	29/68	60/73	16/36	55/75	22/39
27		94/96	24/65	90/93	47/60	76/87	15/58	93/99	36/62	86/85	25/68
46		90/95	15/54	81/89	25/44	63/80	8/46	84/94	18/51	73/83	21/45
73		78/94	.	79/90	.	58/80	.	83/94	.	71/81
20	8·4	99/104	34/72	94/99	57/64	76/83	24/59	90/94	49/67	91/93	37/41	69/78	30/37	41/58	38/37
27		81/92	19/60	90/90	32/50	69/75	11/48	84/91	26/52	89/89	30/29
48		73/84	6/49	84/87	15/34	52/67	8/44	75/84	11/36	87/82	30/20
73		52/79	.	76/82	.	36/61	.	63/81	.	73/74

for contaminating the protoplasts with added lysozyme. Since *Staphylococcus aureus* (strain Duncan)—which had already been the object of osmotic studies—appeared to behave typically among the seven organisms tested, we investigated the factors which determine the rate of development of osmotic fragility in this organism.

Effect of pH value

Fig. 1 shows the dependence of the rate of development of osmotic fragility on the pH value, measured by the rate of change of *a/b* in suspensions of *Staphylococcus aureus* (strain Duncan), harvested at a density corresponding to *c.* 2 mg. dry weight/ml. from rotated flask cultures, and incubated at 25° in 1·2 M-sucrose containing 0·2 M-NaCl, buffered with 0·01 M-(sodium acetate/acetic acid) (–○–) or with 0·01 M-sodium phosphates (–●–) and containing 0·01 M-sodium iodoacetate to prevent acid production and drift of pH. The presence of iodoacetate was shown to be without effect on the rate of development of osmotic fragility (see below). The optimum rate is near pH 6.

Effect of salt concentration

Fig. 2 shows the dependence of the rate of development of osmotic fragility of *Staphylococcus aureus* (strain Duncan) on salt concentration. The measurements were made as above on suspensions incubated at 25° in 1.2 M-sucrose buffered at pH 5.8 with a range of concentrations of acetate buffer containing 9 mole NaAc to 1 mole HAc. The rate is nearly linear with salt concentration up to ionic strength 0.3, at which there is a sharp optimum. Identical results were obtained when the ionic strength was varied by adding NaCl to a constant concentration (0.02 M) of acetate buffer.

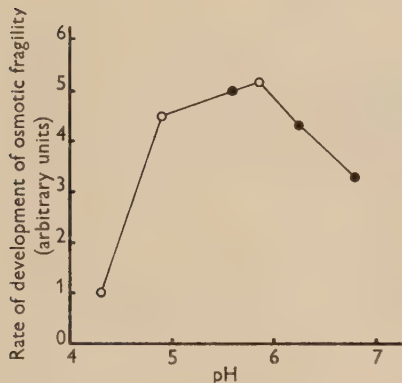


Fig. 1

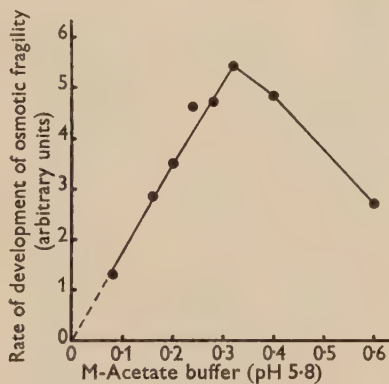


Fig. 2

Fig. 1. Effect of pH on the rate of development of osmotic fragility of *Staphylococcus aureus* (strain Duncan) at 25° in 1.2 M-sucrose containing 0.2 M-NaCl and 0.01 M-sodium iodoacetate buffered with either 0.01 M-(sodium acetate/acetic acid) (—○—) or with 0.01 M-sodium phosphates (—●—).

Fig. 2. Effect of salt concentration on the rate of development of osmotic fragility of *Staphylococcus aureus* (strain Duncan) at 25° in 1.2 M-sucrose buffered at pH 5.8 with a range of concentrations of acetate buffer containing 9 mole NaAc to 1 mole HAc.

Effect of some potential inhibitors and activators

The presence of 0.01 M-sodium ethylenediamine tetra-acetate was found to inhibit by 75 % the rate of development of osmotic fragility of *Staphylococcus aureus* (strain Duncan) in 1.2 M-sucrose containing 0.33 M-acetate buffer at pH 5.8. The addition of $MgCl_2$ or $LaCl_3$ to the autolysis medium at ionic strength 0.3 at pH 5.8 did not cause activation of the development of osmotic fragility nor was the rate affected by the presence of 0.01 M-KCN, 0.01 M-sodium iodoacetate, 0.01 M-sodium thioglycollate or by aeration.

Effect of age of culture

Fig. 3 shows the time course of the logarithm of the dry-weight concentration of *Staphylococcus aureus* (strain Duncan) growing at 25° in a rotated flask, and the rate of development of osmotic fragility of the organisms of corresponding age incubated at 25° in 1.2 M-sucrose containing acetate buffer at pH 6.8 of ionic strength 0.3. The rate of development of osmotic fragility was

in this case measured by the apparent time taken for half the organisms to become osmotically fragile (when $a/b = 0.5$): this being determined from plots of the time course of a (—●—) and b (—○—) such, for example, as that shown in Fig. 4 for the organisms harvested at a concentration corresponding to 0.95 mg. dry weight/ml. For mean generation times shorter than 2 hr. the rate of development of osmotic fragility rises approximately linearly with the rate of growth, but for mean generation times longer than 2 hr. the rate of appearance of osmotic fragility approaches zero. Rapid growth is therefore

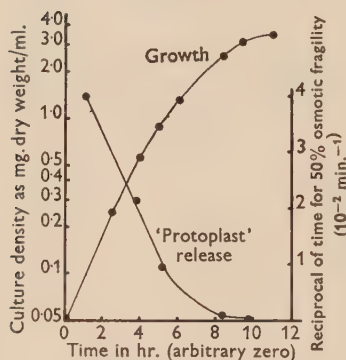


Fig. 3

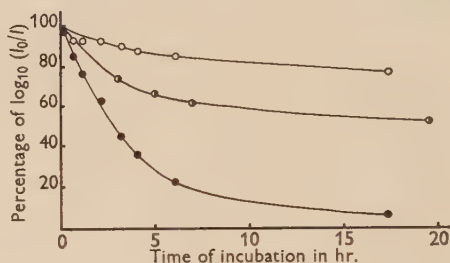


Fig. 4

Fig. 3. Time course of growth of cultures of *Staphylococcus aureus* (strain Duncan) at 25° as mg. dry weight of organisms/ml. on logarithmic scale, with plot of rate of development of osmotic fragility in organisms of corresponding age on incubation at 25° in 1.2 M-sucrose containing acetate buffer of ionic strength 0.3 at pH 5.8.

Fig. 4. Time course of development of osmotic fragility of *Staphylococcus aureus* (strain Duncan) harvested at a concentration corresponding to 0.95 mg. dry weight/ml., and of autolysis of cell walls of organisms of the same age. Organisms incubated at 25° in 1.2 M-sucrose containing acetate buffer of ionic strength 0.3 at pH 6.8. The osmotic fragility is given by percentage initial turbidity of 0.1 ml. samples of incubated suspension pipetted into 5 ml. samples of (a) 0.01 M-phosphate buffer alone at pH 6.8 (—●—) and (b) the same buffer containing 1.2 M-NaCl (—○—). The isolated cell walls were incubated in the same medium as whole organisms and degree of autolysis given by turbidity of samples corresponding to (a) above (—●—).

a prerequisite for the rapid development of osmotic fragility, the time taken for 50% of the organisms from the exponential phase of growth to become osmotically fragile being only 25 min. under the conditions of our experiments.

Internal osmotic pressure

The 'protoplasts', obtained from organisms harvested at a concentration corresponding to c. 1.1 mg. dry weight/ml., by incubation in 1.2 M-sucrose containing 0.33 M-acetate buffer at pH 5.8 at 25° for 20 hr. were generally found to lyse at a rate not greater than 5%/hr. when 0.1 ml. samples were pipetted into 5 ml. samples of 1.2 M-NaCl at 20°. These 'protoplasts' were considered to be suitable for osmotic studies. Fig. 5 (—○—) shows the turbidity

of the 'protoplasts' suspended in a range of concentrations of NaCl buffered with 0.01 M-sodium phosphate at pH 6.8. As in the case of the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* (Mitchell & Moyle, 1956*b*) the 'protoplasts' of *Staphylococcus aureus* require an external concentration of c. 0.7 M-NaCl to stabilize half of them and, since they are not stabilized by 1.2 M-glycerol (Fig. 5, —●—), it is evident that the mean internal osmotic pressure must be c. 20 atmospheres, the internal medium of the organisms being separated from the suspension medium by a semipermeable plasma membrane, permeable to glycerol but not to sucrose and NaCl.

Respiration of 'protoplasts'

The Q_{O_2} for the intact organisms and 'protoplasts' were measured at 25° in 1.2 M-sucrose containing 0.33 M-acetate buffer at pH 5.8 in Warburg manometers as recommended by Dixon (1934). A typical batch of the normal organisms harvested at a density corresponding to 1.42 mg. dry weight/ml. gave Q_{O_2} values of 9.6 for residual respiration and 7.5 for glucose oxidation,

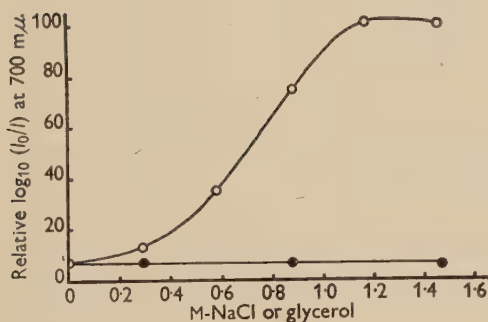


Fig. 5

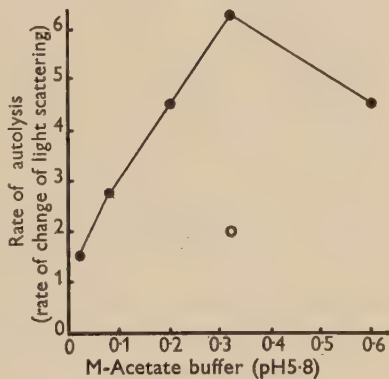


Fig. 6

Fig. 5. Dependence of stability of 'protoplasts' of *Staphylococcus aureus* (strain Duncan) on the concentration of NaCl (—○—) and of glycerol (—●—), buffered at pH 6.8 with sodium phosphates, indicated by the turbidity of the suspensions expressed as a percentage of the value in 1.2 M-NaCl.

Fig. 6. Dependence of rate of autolysis of cell walls of *Staphylococcus aureus* (strain Duncan) on salt concentration measured by the rate of change of turbidity, under conditions corresponding to those of Fig. 2 for the intact organisms (—●—). The isolated point (○) shows the effect of the presence of 0.01 M-sodium ethylenediamine tetra-acetate.

determined immediately after harvesting and washing. The 'protoplasts' prepared from these organisms by treatment as in the above paragraph for 40 hr. gave Q_{O_2} values of 9.6 for residual respiration and 6.3 for glucose oxidation. Thus, the residual respiration of intact organisms and 'protoplasts' is the same but glucose oxidation is depressed to 84 % of its normal value in the 'protoplasts'. We may therefore infer that in spite of the prolonged autolytic treatment the enzyme systems of the 'protoplasts' remain virtually intact.

Autolytic systems of the cell walls

Since the development of fragility of the organisms must depend on loss of tensile strength of the cell wall, it was reasonable to suppose that osmotic fragility might be due to digestion of cell wall by an autolytic system within the organism. Attempts to obtain an autolytic system from the soluble fraction of *Staphylococcus aureus* (strain Duncan) after mechanical disintegration and fractionation as previously described (Mitchell & Moyle, 1951) were unsuccessful. However, the cell-wall fraction was found to autolyse under the same conditions and at about the same rate as whole organisms. The rate of autolysis of cell walls obtained from a culture harvested at a density corresponding to 0.95 mg. dry weight/ml. is shown in Fig. 4 (—●—) by the turbidity plotted as a fraction of the initial value, the conditions of incubation being identical to those used for the intact organism described in the same figure. Fig. 6 shows the dependence of the rate of autolysis of the cell walls (measured by rate of change of turbidity) on salt concentration under conditions corresponding to those of Fig. 2. At ionic strength 0.3, 0.01 M-sodium ethylenediamine tetra-acetate depressed the rate of autolysis to a value close to that corresponding to zero ionic strength (Fig. 6). The autolysis of the isolated cell-wall fraction was found to be unaffected by repeated washing at 2° with distilled water or with 0.2 M-NaCl, 0.2 M-KCl or 0.2 M-sodium acetate buffer at pH 5 or pH 5.8.

Autolysates of the cell wall of *Staphylococcus aureus* contained a factor which would lyse intact *Micrococcus lysodeikticus* (NCTC 2665) and dissolve the cell-wall fraction of old cultures of this organism (prepared as that of *S. aureus*) in 0.02 M-sodium phosphate buffer at pH 6.8. It was also observed that *M. lysodeikticus*, harvested during the exponential phase of growth, became osmotically fragile and that the cell-wall fraction of organisms of this age autolysed under conditions similar to those applying to *S. aureus*.

A more detailed study of the lytic factor of the cell walls of *Staphylococcus aureus* and its possible relationship to lysozyme was not undertaken since it was not within the scope of the present work to characterize the system responsible for the autolysis of the cell wall but only to discover the optimal conditions for its operation. It was, however, pertinent to consider in more detail the mechanism of development of osmotic fragility from a morphological point of view.

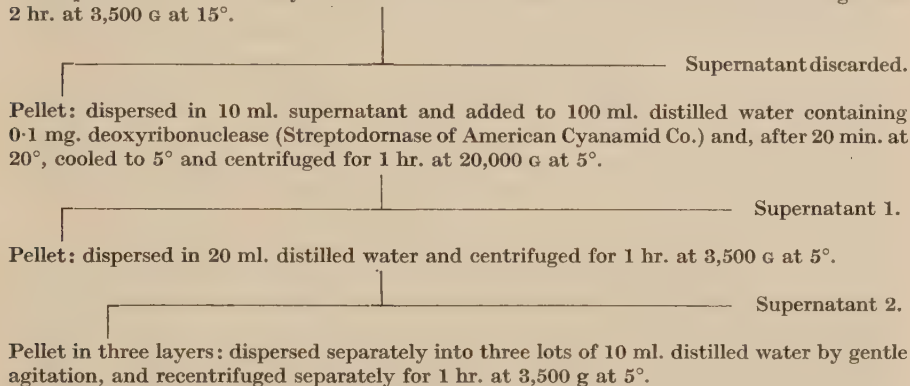
Morphological changes accompanying osmotic fragility

There are three obvious types of change which might give rise to osmotic fragility: (i) the wall might be completely removed from the surface of the plasma membrane; (ii) the wall might undergo a change of structure or cross-linkage which would cause a uniform loss of tensile strength over its surface without appreciable change of weight; (iii) the wall might be lost or weakened at one or more particular sites on the surface of the cell. An attempt was made to decide between these alternatives by studying the morphology of the fragments of the cell wall and plasma membrane after exploding the 'proto-

plasts' osmotically. The suspensions which had undergone 'protoplast' release in 1.2 M-sucrose were osmotically exploded and the fragmented organisms were separated at 5° into supernatant, plasma-membrane and cell-wall fractions as described in Table 2. In Table 3 the weights of the fractions recovered from the 'protoplasts' released from organisms harvested

Table 2. *Explosion and fractionation of 'protoplasts' of Staphylococcus aureus (strain Duncan)*

'Protoplasts' in 40 ml. autolysis medium added to 200 ml. 1.2 M-NaCl and centrifuged for 2 hr. at 3,500 G at 15°.



The appropriate layers of the three fractions and their supernatants were recombined and the centrifuging repeated as above (in some cases through 2 further cycles) to give four practically homogeneous fractions as follows:

- (a) Supernatant, combined with Supernatants 1 and 2 above.
- (b) Light fraction, bright yellow (plasma-membrane fraction).
- (c) Intermediate fraction, pure white (cell-wall fraction).
- (d) Heavy fraction, pale yellow (intact-organisms fraction).

at a concentration corresponding to 2 mg. dry weight/ml. are compared with the weights of the same fractions obtained after mechanical disintegration of part of the same batch of washed suspension using the sonic disintegrator of H. Mickle as previously described (Mitchell & Moyle, 1951). Phase-contrast microscopy of the fractions obtained from the autolytic 'protoplast' release showed that the 'plasma-membrane fraction' consisted of spherical shells of very low contrast, identical in appearance to the ghosts obtained from the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* (Mitchell & Moyle, 1956b). When this fraction was washed for electron microscopy it disintegrated and became condensed into particles readily visible by phase-contrast microscopy. This material appeared identical in the electron microscope to the 'small particle fraction' isolated from mechanically disintegrated organisms (Mitchell & Moyle, 1951) and the same total weight of it was obtained by the autolytic and mechanical disintegration methods (Table 3). The 'plasma-membrane fraction' of the autolytic preparation also had the same enzymic composition as the 'small-particle fraction' of the mechanical disintegration method (Mitchell & Moyle, 1956c). We suggested previously (Mitchell & Moyle 1951) that the 'small particle-fraction' of the mechanically disintegrated

organisms represented the plasma membrane of the intact cells because of its staining reactions, its high lipid content and the fact that there is sufficient to form a spherical shell *c.* 50 Å thick and 0.7 μ . in diameter when dry. Since we are now able to recover material of corresponding properties and total weight in the form of spherical shells, there seems to be little doubt that this material represents the plasma membrane.

Table 3. *Weight and morphology of cell envelope fractions collected after mechanical or autolytic disintegration of Staphylococcus aureus (strain Duncan)*

Material	Mechanical treatment		Autolytic treatment	
	Weight (mg.)	Morphology	Weight (mg.)	Morphology
Intact organisms	100	.	100	.
Cell walls	16.6	Spherical shells	12.5	Hemispherical shells
Plasma membranes	9.6	Small particles	10.2	Spherical shells

The weight of cell wall recovered from the use of the autolytic method was 25 % less than from that of the mechanical one (Table 3), and the electron microscopy showed that whereas the material obtained from the mechanical disintegration consisted of punctured spherical shells (Pl. 1, fig. 1), that obtained from the autolytic method consisted entirely of hemispherical shells (Pl. 1, fig. 2). An examination of some hundreds of cell walls in photographs and on the fluorescent screen of the electron microscope showed that whereas the splits in the walls from the mechanically disintegrated organisms did not appear in any particular position relative to the equatorial ring (when visible), the edge of the autolytically produced hemispherical shells were invariably at right angles to the equatorial ring (when visible). Further, a pair of hemispheres was never seen to be joined by part of the edge. It therefore seemed reasonable to infer that the autolytic treatment resulted in the loss of an equatorial ribbon of cell wall, leaving the cell contents (in 1.2 M-sucrose or other medium of equivalent osmotic pressure) as a protoplast capped by two mechanically separate hemispheres of cell wall.

It is necessary to emphasize that the fractionation of the osmotically exploded organisms described above was carried out on organisms harvested from cultures at a concentration corresponding to *c.* 2 mg. dry weight/ml. We have not so far been successful in obtaining satisfactory fractionation of osmotically exploded organisms from cultures harvested during rapid growth; perhaps the autolysis of the cell wall of younger organisms is not so strictly localized in an equatorial ribbon as in older ones. On the other hand, it is possible that after osmotic explosion the plasma membranes tend to adhere to the hemispherical caps of cell wall more in young than in old organisms.

DISCUSSION

The word 'protoplast' may conveniently be applied to the osmotically fragile staphylococci in which the cell wall has lost its tensile strength, but it would not be legitimate to remove the inverted commas except in cases where it can

be shown that practically speaking none of the cell-wall material is still attached to the surface of the protoplast.

A number of studies on the autolytic systems of Gram-positive bacteria have indicated the presence of lysozyme-like enzymes. For instance, Meyer, Palmer, Thompson & Khorazo (1936) observed the production of a lysozyme-like factor by *Sarcina* sp., Webb (1951) found a similar factor in *Clostridium welchii* and Greenberg & Halvorson (1955) found that lysozyme-like lytic enzymes were released from autolysing *Bacillus cereus* and *B. terminalis*. Recently, Richmond (1956) isolated and partially purified a lytic enzyme from an aerobic spore-forming rod which resembles egg-white lysozyme closely but is not identical with it. Stähelin (1953) observed autolytic spherical transformation in a particular strain of *Bacillus anthracis* in Ringer’s solution.

It is possible that the main component of the autolytic systems which attack the cell walls of the cocci described in the present work is a lysozyme-like enzyme which acts on the same linkages in the cell wall as are attacked by egg-white lysozyme. Further work will be required to establish whether this may be true or whether other enzymes are mainly or partially implicated.

It is interesting that the ribbon of cell wall which appears to be digested or weakened by the autolytic system of *Staphylococcus aureus* (strain Duncan) should lie at right angles to the equatorial ring; for, since the successive planes of division in staphylococci lie at right angles, one might infer that the digested or weakened ribbon of wall corresponds to the division before (or possibly after) that represented by the equatorial ring.

The question arises as to the function of the autolytic systems in the growing organisms, since the autolytic activity is greater the greater the rate of growth. We can suggest two possible functions: (i) the lytic enzymes may normally have a synthetic function like, for instance, the enzymes synthesizing or degrading the glutamic acid polypeptides of the capsules of *Bacillus subtilis* and *B. anthracis* studied by Thorne (1956); (ii) the lytic enzymes may normally act as hydrolytic elements in the system responsible for the change of shape and disengagement of the cell walls of the dividing bacteria. In either case the activity of the lytic system would be expected to be greater the more rapid the rate of growth.

The release of the protoplasts of staphylococci from the mechanical protection of the cell wall makes possible the isolation and characterization of the plasma-membrane material and facilitates the study of the internal osmotic pressure and permeability properties of these organisms.

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EXPLANATION OF PLATE

- Fig. 1. Electron micrograph of cell-wall fraction of mechanically disintegrated *Staphylococcus aureus* (strain Duncan), shadowed at an angle of 15° with gold-palladium. $\times 16,000$.
- Fig. 2. Electron micrograph of cell-wall fraction of autolytically disintegrated *Staphylococcus aureus* (strain Duncan), shadowed at an angle of 15° with gold-palladium. The edges of the hemispherical shells lie at right angles to the equatorial ridges when visible. $\times 16,000$.

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P. MITCHELL & J. MOYLE—'PROTOPLASTS' FROM *STAPHYLOCOCCUS AUREUS*. PLATE 1
(Facing p. 194)

The Structure of Influenza Virus Filaments and Spheres

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SUMMARY: Influenza virus filaments treated with acid on an electron microscope film developed along their length rows of spheres which were completely digested by trypsin. Influenza virus spheres similarly treated revealed trypsin-resistant polygonal rings which by their behaviour with enzymes have been identified as ribonucleoprotein.

The demonstration by Mosley & Wyckoff (1946) and by Chu, Dawson & Elford (1949) that influenza virus could occur in long filaments as well as in the more usual spherical forms was followed by many investigations into, and theories about, the nature of the two forms and their relation to one another. Theories have been based on study of the conditions under which filaments are formed and on the biological properties of filaments and spheres. In the present investigation, we have compared the morphology of filaments and spheres, after different chemical treatments, as shown by the electron microscope. The results emphasize striking structural differences between filaments and spheres, and at the same time allowed us to examine the gross structure of the influenza virus nucleoprotein.

METHODS

Viruses. Two strains of influenza virus A were investigated. The MEL (1935) strain contains many spheres and only a very small proportion of filamentous forms. The A/Persia/2/52 virus which has had only a few passages in hen eggs produces roughly equal numbers of filaments and spheres. Both strains were propagated by passage in the allantoic cavity of 10-day chick embryos. The virus was prepared for electron microscopy by adsorbing on to chick red cells at 0° and eluting into saline at 37°; it was usually concentrated about fivefold by this technique.

Trypsin. Crystalline trypsin (Armour) was kept as a 4% (w/v) solution in 0.01 N-HCl at -10°. Before use it was diluted in phosphate buffer (pH 8.0) to give a 0.1% solution and centrifuged lightly.

Ribonuclease. Crystalline ribonuclease prepared from beef pancreas by the method of Kunitz (1940) was kindly supplied by our colleague Dr R. R. Porter. In one experiment a preparation of three times crystallized ribonuclease was used. The ribonuclease was made up in phosphate buffer (pH 8.0) at a concentration of 0.1% (w/v).

Electron microscope techniques. The film supports used were made of platinum. A very thin film of nitrocellulose was used to cover them, stabilized by evaporating on to it the thinnest possible film of carbon, an adaptation of the method described by Bradley (1954).

A drop of the virus suspension in saline was placed on each film for 1 min. and then washed away by immersing it in distilled water for 1 min. Many of the virus particles that impinge on the film are held there by short range inter-molecular forces and do not then wash off. The platinum supports with the virus thus adsorbed on the films were placed in the various reagents to be described, with a brief wash in distilled water between treatments. The structures on the films were then fixed with osmium tetroxide vapour for 5 min., stained by immersion for 5 min. in a 5% (w/v) solution of phosphotungstic acid (Hall, 1955) and then finally washed in distilled water. After lightly blotting at the edge with filter-paper, the preparations were allowed to dry. From the moment the virus suspension had been placed on the film until after this final washing no drying had occurred; this formed an important part of the technique. The phosphotungstic staining was used to replace the more usual metal shadowing in all experiments but one. The superiority of this staining technique for revealing underlying structures was at once apparent.

The preparations were viewed in a Siemens ÜM 100 electron microscope using an anode potential of 60 kV. Plates were taken at a magnification of either 14,000 or 55,000 times.

RESULTS

Morphology of the virus after chemical treatments

Treatment of filaments and spheres with the virus adsorbed on the film allowed us to carry out a series of consecutive treatments with different reagents while the virus remained *in situ* and in a good condition for electron microscopic observation. A mixture of partially purified MEL and A/Persia/2/52 viruses was used in order to have a high concentration of spheres and filaments on the same electron microscope film, and therefore treated under identical conditions. This filament + sphere mixture was examined after the following consecutive treatments:

- (1) Untreated control.
- (2) 0.1 N-HCl. Treatment was carried out at room temperature.
- (3) 0.1% trypsin at 37°.
- (4) 0.1% ribonuclease at 37°.
- (5) A second treatment with 0.1 N-HCl and 0.1% trypsin.

In the usual experiment, five specimens were prepared, along with the additional controls which are considered later, and at the end of each stage in the treatment one was washed, fixed and stained with phosphotungstic acid. The results to be described summarize the observations on many experiments of this kind in all of which the appearances at each stage were quite consistent.

(1) *The untreated suspension.* Untreated suspensions were examined after fixation in osmium tetroxide vapour and impregnation with phosphotungstic acid as described under Methods. Electron micrographs showed the virus elementary bodies as roughly spherical with a diameter of about 800 Å. and the filaments with a more or less uniform width of about 650 Å. and a very

variable length ranging from 2,000 Å. up to 50 μ . The spheres were normally more electron dense than the filaments (Pl. 1, fig. 1). Running along the length of the filaments could be seen structures which on close examination appeared as dense threads about 50 Å. thick (Pl. 1, fig. 2). The spheres seemed to have a similar structure but the threads were denser and rather thicker than in the filaments and packed together more tightly. For this reason they were seen less clearly, many of the spheres appearing almost homogeneously dense (Pl. 1, fig. 3). Examination of the filaments never revealed spheres within them but spheres were often seen attached to their sides and tips (Pl. 1, fig. 1).

(2) *Treatment with hydrochloric acid.* While 30 min. in 0.1 N-HCl did not obviously alter the appearance of the virus spheres, 30 sec. in this acid produced striking changes in the filaments (Pl. 1, figs. 4, 5). They characteristically broke up into strings of spherical bodies, some swollen to several times the diameter of the original filament, while others bore a very remarkable resemblance to virus spheres. It is not easy to believe that this resemblance was purely coincidental, but the results described in the next section point to a fundamental difference between the true virus spheres and those produced in filaments by acid treatment.

(3) *Treatment with trypsin.* By itself trypsin produced little change in either filaments or spheres (2 hr. treatment with 0.1 % trypsin, pH 8.0, at 37°). But when the preparation of virus particles on the film was first treated with 0.1 N-HCl for 5 min. and then transferred to the trypsin, considerable digestion rapidly took place, 5 min. in the enzyme being sufficient to remove almost all traces of the filaments. The spheres were left frequently as irregular ring-like forms and after about 1 hr., the digestion had gone about as far as possible (Pl. 3, fig. 11), since the appearances remained the same throughout a further 4 hr. in trypsin. Not all the trypsin-treated spheres showed ring forms, but in those which did not, appearances were often consistent with those of intact or broken rings viewed at different angles.

Examination of the structures left after digestion with trypsin showed no continuous resistant structure of resolvable size running along the length of the filaments. A considerable part of the structure of the virus spheres resisted digestion but after treatment of the mixture of filaments and spheres these remains were never obviously arranged in chains showing that trypsin-resistant structures do not occur periodically along the filaments. This does not rule out the possibility that an occasional point along the filaments might have resisted digestion but unlike the spheres, the filament structure was mostly trypsin-sensitive.

The two most notable features of the spheres after treatment with acid and trypsin were first that the resistant structures appeared typically as rings (often suggestively polygonal in outline) with no evidence of any resistant central portion (Pl. 2, figs. 6-8), and secondly that the structures sometimes expanded up to several times the diameter of the original virus sphere (Pl. 2, fig. 9). A double line of staining separated by a clear space about 40 Å. wide could be seen in some places running round the ring of the resistant structure. It is well shown in Pl. 2, fig. 9, where the structure had opened right out

and the ring broken, but it was also commonly observed in the smaller rings.

When, instead of the treatment with phosphotungstic acid, the preparations were shadowed with platinum, the trypsin-resistant structures cast very definite shadows and in some cases they must have represented up to 30 % by volume of the original particles (Pl. 2, fig. 10).

(4) *Treatment with ribonuclease.* Preparations treated with acid and trypsin were then rinsed briefly in distilled water and transferred to a solution of ribonuclease (0.1 % crystalline ribonuclease, pH 8.0, for 80 min. at 37°). Examination after fixing and staining showed that digestion of the virus structure had proceeded no further as judged by morphological appearance after this further treatment (Pl. 3, fig. 12). The ribonucleic acid content of the virus spheres has been determined as about 0.8 % by weight of their total mass (Ada & Perry, 1956) and this nucleic acid would be expected to form a part of the trypsin-resistant structure. Evidence that the enzyme had really acted on the resistant structures despite the lack of any marked morphological change was provided by the treatment now described.

(5) *Second trypsin treatment.* Following the treatment with acid, trypsin and ribonuclease, films were then further treated with acid for 5 min. and trypsin for 80 min. under the same conditions as before. When these films were fixed and stained, only a very occasional trace of any structures was to be found (Pl. 3, fig. 13). It was therefore concluded that the structures resistant to the first treatment with trypsin were composed of protein closely linked with ribonucleic acid and only susceptible to proteolytic digestion after the nucleic acid had been attacked by the nuclease.

Control observations. Virus particles were removed from the suspension by adding fowl red blood cells on to which the virus was adsorbed, followed by light centrifugation to spin out the cells. The haemagglutination titre of the virus suspension used was reduced in this way by 99.6 % and when examined in the microscope only a very few particles could be found. These preparations treated with acid and trypsin showed no structures similar to those seen when the unadsorbed suspensions were treated in this way.

When the virus particles were immersed first in trypsin and then in acid, the result was exactly like that produced by acid alone, confirming that trypsin had little effect on the spheres or filaments unless they were first treated with acid.

Virus particles were treated with ribonuclease and then with acid followed by trypsin, but the result was the same as without ribonuclease; this enzyme thus acted only after the particles had first been attacked with trypsin. By itself, ribonuclease had no effect on the appearance of the virus particles.

As described above, the virus was entirely digested by the sequence of treatments: 0.1 N-HCl (5 min.), 0.1 % trypsin (80 min.), 0.1 % ribonuclease (80 min.), 0.1 N-HCl (5 min.), 0.1 % trypsin (80 min.). The same preparation was treated in parallel but with omission of the ribonuclease and extension of the trypsin treatment, viz. 0.1 N-HCl (5 min.), 0.1 % trypsin (160 min.), 0.1 N-HCl (5 min.), 0.1 % trypsin (80 min.). The result was that usually

observed after acid and trypsin, the typical resistant structures of the spheres still being present.

A preparation of 'incomplete' (von Magnus, 1946) MEL virus was treated with acid and trypsin as described above, but the ring structures were similar to those seen after treating standard MEL virus. 'Incomplete' virus has a lower ribonucleic acid content than standard virus (Ada & Perry, 1956), but unfortunately we have had difficulty with the MEL strain of virus in preparing 'incomplete' virus with less than one-hundredth of the relative infectivity of standard virus. According to Ada & Perry (1956) this virus should have half the concentration of ribonucleic acid of standard virus. It is possible that 'incomplete virus' with an even lower ribonucleic acid content than this might show structural differences from standard virus after treatment with acid and trypsin, and further experiments along this line are contemplated.

Pepsin. Treatment with pepsin (0.1 % pepsin in 0.1 N-HCl for 1 hr. at 37°) showed less digestion than treatment with trypsin, the most obvious difference being the effect on the filaments. These had ballooned at points, presumably because of the hydrochloric acid, but much of their structure remained clearly identifiable, in striking contrast with the results after treatment with trypsin. After pepsin treatment, many of the spheres showed resistant rings of material similar in appearance to the structures resistant to trypsin, but again the digestion had gone less far.

Effect of water on the virus

Burnet (1956) showed that influenza filaments became beaded and then disintegrated in distilled water without, however, any significant fall occurring in the infectivity of the preparation. We confirmed that filaments of the Persia strain adsorbed on the film showed a marked change in appearance after immersion in distilled water for 1 hr. (Pl. 3, fig. 14). The filaments were mostly ballooned into a series of disk-like forms. The spheres appeared unaffected by this treatment but they too were not always able to withstand completely salt-free distilled water for long. Pl. 3, fig. 15, shows the striking appearance of a suspension of the MEL strain, initially in saline, after overnight dialysis against 300 times its volume of distilled water followed by 4 hr. further dialysis against a similar volume of fresh distilled water. The spheres had then obviously started to break down and showed thread-like structures often forming small polygonal shapes, the threads extending outwards from the spheres.

Further observations on the occurrence and behaviour of the filaments

Occurrence. Short filaments occur in a number of viruses of the mumps-influenza (*Myxovirus*) group, e.g. influenza B and C and fowl plague, but abundant long filaments are characteristic of recently isolated strains of influenza virus A. We have found that filament production is a property which is independent of the O-D phase behaviour of the virus (Burnet & Bull, 1943) and of the 'incompleteness' of the virus (von Magnus, 1946). A strain of influenza virus A was isolated in monkey kidney in the 'O' (or original) phase (Burnet & Bull, 1943); when examined in the electron microscope it showed

typical virus filaments which could be adsorbed on guinea-pig red cells. Also, 'incomplete virus' prepared from the A/Persia/2/52 virus by the technique of von Magnus (1951) had an infectivity/agglutinin titre ratio 10^{-3} that of standard virus; the appearance of this virus in the electron microscope was indistinguishable from that of standard virus.

Infectivity. The experiments of Donald & Isaacs (1954) suggested that influenza virus filaments were about as infectious as spheres, but since a pure preparation of filaments had not been obtained it was difficult to exclude with certainty the possibility that filaments were not infectious. We have recently found that the extremely long filaments present in infected allantoic fluid tended to break when adsorbed on red cells; this was evident from the fact that although the very long filaments were removed from a virus preparation by absorption with red cells, no filaments as long were to be seen on these cells. Presumably they broke during the pipetting needed to redisperse the centrifuged red cells. This means that the counts of filaments made by Donald & Isaacs (1954) were probably over-estimates of the true number of filaments present, and hence that there is an increased probability (which unfortunately cannot be expressed quantitatively) that the filaments are infectious. In an attempt to prepare a pure preparation of filaments we tried to filter the spheres through collodion membranes of average pore diameter 260 and 990 m μ . in order to see whether the filaments would be retained above the membrane. Repeated washings were carried out in this way, but the 260 m μ . membrane kept back the same proportions of filaments and spheres, while the 990 m μ . membrane adsorbed all the filaments in the filter pores.

The finding of Chu *et al.* (1949) that filaments can be agglutinated by a convalescent ferret antiserum was confirmed by electron microscopic examination (Bang & Isaacs, 1956). This is further evidence that the filament has virus-specific antigen along its surface.

DISCUSSION

The structure of an influenza virus filament suggested by this technique is a row of spheres which are acid-resistant but trypsin-sensitive after acid treatment, linked by some acid-soluble material. The spherical structures produced in the filaments by acid treatment are presumably protein containing the viral haemagglutinin. The alternative is that the filament contains haemagglutinin distributed uniformly along its surface and that on treatment with acid this becomes bunched into spherical bodies. The need for acid treatment before digestion occurs with trypsin may be due to the greatly increased rate of digestion of globular proteins that follows their denaturation (Haurowitz, Tunca, Schwerin & Goksu, 1945), the acid exposing the vulnerable links in the protein chain to the action of the enzyme. Another possibility is that the acid may be necessary to remove a protective coating from around the virus particle.

It appears that intact virus spheres are made of an outer protein coat, which includes the viral haemagglutinin, around a ribonucleoprotein structure; the

idea that the ribonucleoprotein is enclosed within a protein coat is based on the fact that ribonuclease will not act until the spheres have been first treated with acid and trypsin. If the infectivity of the virus is closely bound up with its nucleic acid, as is suggested by the results of Ada & Perry (1956), the ribonucleoprotein structure is probably the essential replicating part of the virus, and it seems that filaments do not have these structures occurring along their length. Possibly each filament has a single ribonucleoprotein structure at one end, but since filaments are very rapidly digested by trypsin, and since it has not been possible to obtain a pure preparation of filaments we have been unable to verify this possibility. The suggestion, however, would be compatible with the findings (Donald & Isaacs, 1954) that on treatment with ultrasonic vibrations, influenza virus filaments broke up into numerous rods and there was a big rise in the haemagglutinin titre but no change in the virus infectivity; influenza virus spheres similarly treated showed no change in viral haemagglutinin or infectivity. Studies of the nucleic acid content of filaments, which are now in progress, may help to clarify this picture.

The ribonucleoprotein structure found within the sphere has been of great interest. From shadowed preparations it appears to make up a substantial proportion of the intact sphere, possibly up to about 30 %. The ribonucleoprotein structure did not disappear following treatment by ribonuclease, but only when ribonuclease treatment was followed by a second trypsin treatment. If the ribonucleoprotein is about 30 % of the mass of the viral sphere, then the ribonucleic acid, which forms about 0.8 % of the intact sphere is only about 2.5 % of the ribonucleoprotein structure. The ribonucleic acid is intimately bound up with the protein in such a way that the protein is resistant to tryptic digestion until this comparatively small amount of nucleic acid has been first attacked by ribonuclease, but there is no evidence of how the nucleic acid may be arranged in the ribonucleoprotein. The double line of staining seen very clearly in Pl. 2, fig. 9, is apparently not an artefact since in preliminary experiments a corresponding nucleoprotein ring found in Newcastle disease virus of fowls showed a similar but treble line of staining, but the experiments do not indicate whether those lines are due to nucleic acid or to protein.

A striking feature of the ribonucleoprotein structure has been the frequency with which it has shown a polygonal outline. Many of the polygons seem to be pentagons viewed from different angles, but it is difficult to be sure that they are not hexagons with one side foreshortened. The question arises, what is the structure of the nucleoprotein in the intact virus sphere? Three possibilities may be considered. It may occur as a polygonal ring lying near the circumference of the virus sphere, or as a long thread coiled up within the sphere which uncoils to form a ring when the surrounding protein is digested away, or the structure may be a hollow sphere collapsed in its centre to give a ring-like appearance. The last suggestion seems the least likely from an examination of many pictures, and it does not account for the frequent finding of polygons. The first suggestion, that a polygonal ring occurs within each virus sphere would seem the most probable, but intact virus spheres have given little indication of any such internal structure. It is still necessary to account for the

fact that some of the ribonucleoprotein structures are very much larger than the original virus sphere. Possibly the nucleoprotein has some sort of folded, coiled or helical substructure which is capable of uncoiling under certain conditions, and the way in which it is attached to the film might decide whether uncoiling could occur.

Preliminary work on fowl plague virus and the virus of Newcastle disease of fowls has shown that they, too, are digested to give trypsin-resistant rings. Schafer & Zillig (1954) have shown that fowl plague virus can be fractionated by ether into two components. One, termed the 'haemagglutinin', consists of spherical bodies 300 Å. in diameter and the other, the 'bound antigen', of rods 150 Å. wide and up to 1,000 Å. long. This bound antigen was found by chemical analysis to be a ribonucleoprotein and the electron micrographs published by these authors suggest that their rods might well be broken sides of the trypsin-resistant rings we have just described. Frisch-Niggemeyer & Hoyle (1956) have similarly fractionated influenza A virus and found that the entire ribonucleic acid content of the virus is combined with protein in their 'soluble antigen' fraction. It would be worth while to see whether the 'haemagglutinin' fraction of these viruses was sensitive to treatment with acid and trypsin, and the ribonucleoprotein to treatment with ribonuclease and trypsin.

In contrast with this picture of the nucleoprotein of the influenza virus is the picture now well established for the pox group of viruses (Dawson & McFarlane, 1948; Peters & Stoeckenius, 1954). These viruses contain deoxyribonucleic acid and when digested with pepsin show a central resistant 'nucleus' cuboid in form. The central body can be digested away only when treated with deoxyribonuclease followed by treatment with further pepsin (Peters & Stoeckenius, 1954)—a procedure analogous to that described here using ribonuclease and trypsin for the influenza nucleoprotein. It would be of great interest to know whether the animal viruses which contain ribonucleic acid differ in the gross structure of their nucleoprotein from those which contain deoxyribonucleic acid and this possibility is now under study.

Liu (1955) found by means of fluorescent-labelled antibody that influenza virus 'soluble' antigen, presumably the ribonucleoprotein, was first detectable in the nucleus and later in the cytoplasm of the infected cells of ferret nasal turbinates, whereas the virus specific antigen, i.e. the haemagglutinin, was only found close to the cell surface. We may speculate that the nucleoprotein structures which we have demonstrated are formed in the cell nucleus and pass into the cytoplasm towards the cell surface where they induce the formation of viral haemagglutinin. If the infecting virus is poorly adapted to growth in the particular cells, it may not interfere with the formation of microvilli by these cells. As a result virus may be excreted from the cells to a large extent in the form of long filamentous processes representing microvilli which have been converted into virus filaments (Bang, 1955). If the infecting virus is well adapted to the cells it may so upset the cell surface activity as to inhibit the formation of microvilli with the result that the virus is excreted mainly as ribonucleoprotein rings incorporated in spheres of haemagglutinin. This hypothesis is based on scanty evidence but it may stimulate experiments to see

whether by suitable treatment cells can be encouraged to produce increased numbers of filaments or spheres.

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EXPLANATION OF PLATES

PLATE I

Preparations fixed in osmium tetroxide vapour and then treated with phosphotungstic acid.

Fig. 1. Untreated preparation of influenza virus filaments and spheres. $\times 20,000$.

Fig. 2. Part of a filament. $\times 120,000$.

Fig. 3. Influenza virus spheres. $\times 120,000$.

Fig. 4. Filaments and spheres after treatment with 0.1 N-HCl. $\times 20,000$.

Fig. 5. Part of a filament after treatment with 0.1 N-HCl. $\times 120,000$.

PLATE 2

Preparations fixed in osmium tetroxide vapour. Figs. 6-9 treated with phosphotungstic acid; fig. 10 shadowed with platinum.

Figs. 6-10. Influenza virus spheres after treatment with 0.1 N-HCl and 0.1 % trypsin. $\times 120,000$.

PLATE 3

Preparations fixed in osmium tetroxide vapour and then treated with phosphotungstic acid.

Fig. 11. Influenza virus filaments and spheres after treatment with 0.1 N-HCl and 0.1 % trypsin. $\times 20,000$.

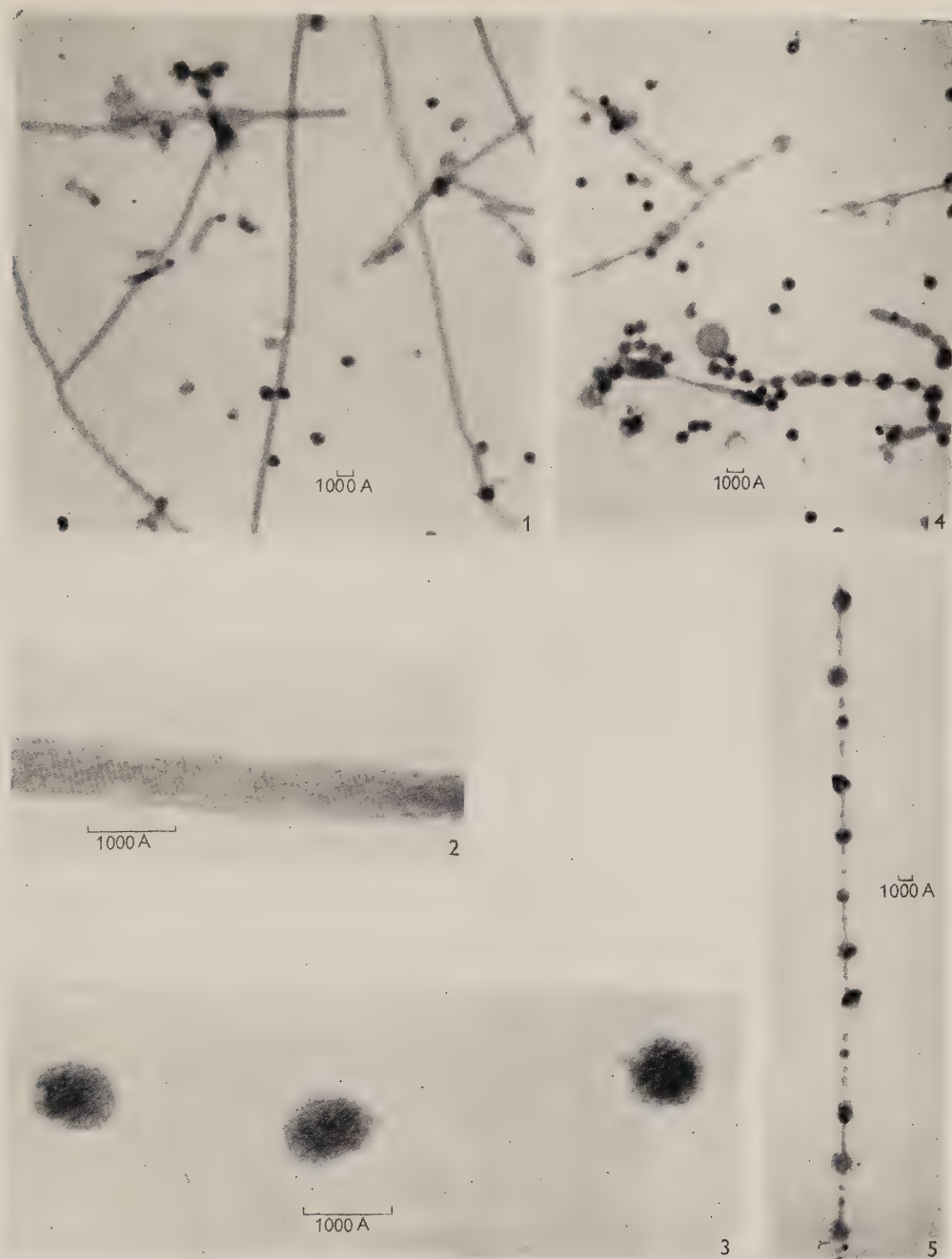
Fig. 12. Influenza virus filaments and spheres after treatment with 0.1 N-HCl, 0.1 % trypsin and 0.1 % ribonuclease. $\times 20,000$.

Fig. 13. Influenza virus filaments and spheres after treatment with 0.1 N-HCl, 0.1 % trypsin, 0.1 % ribonuclease and a second treatment with HCl and trypsin. $\times 20,000$.

Fig. 14. Influenza virus filaments and spheres after immersing in water for 1 hr. $\times 20,000$.

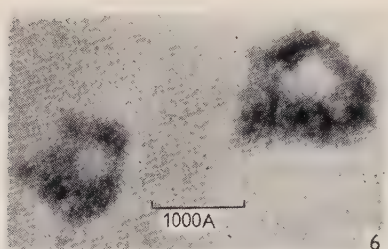
Fig. 15. Influenza virus spheres after dialysis against water. $\times 50,000$.

(Received 11 August 1956)

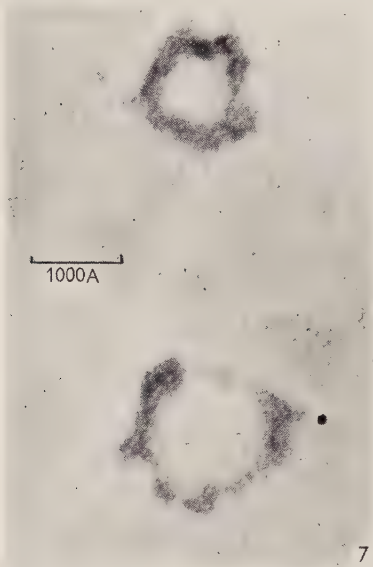


R. C. VALENTINE AND A. ISAACS—STRUCTURE OF INFLUENZA VIRUS. PLATE 1

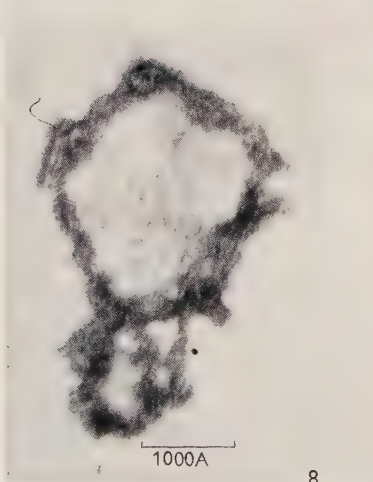
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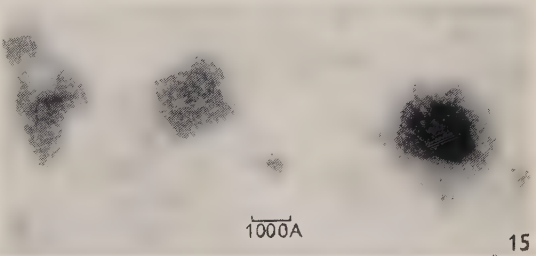
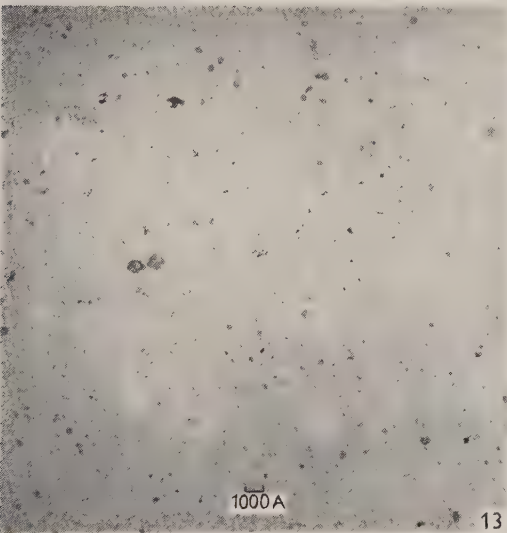
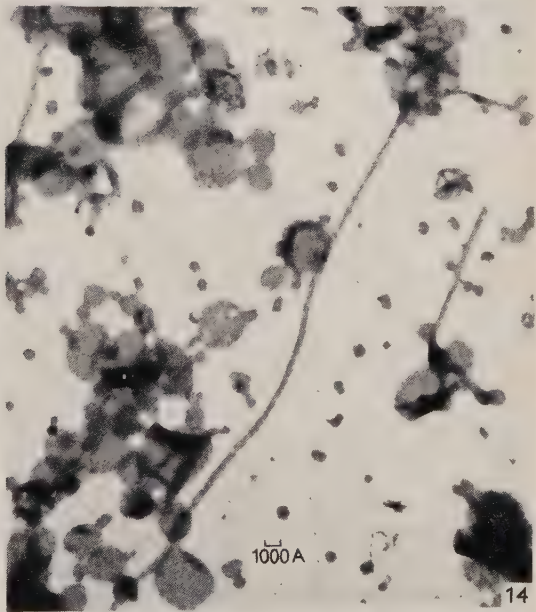
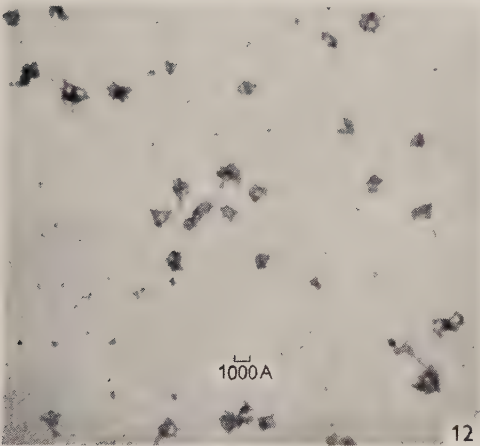
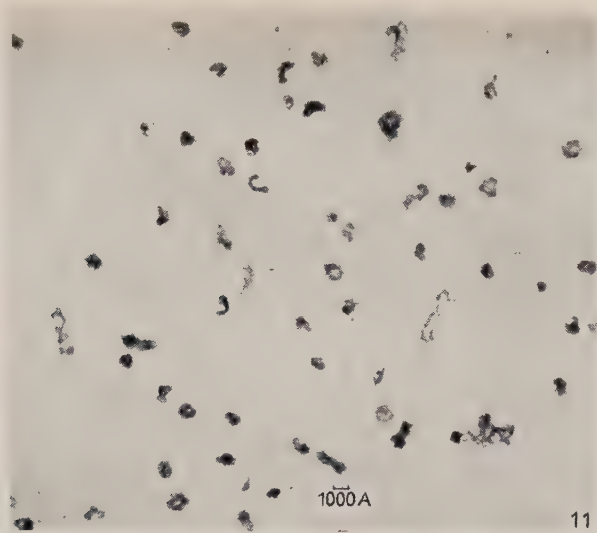
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The Occurrence and Distribution of Bacterial Types on Flatfish

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SUMMARY: An investigation of the bacterial flora of the representative flatfish, skate and lemon sole, was carried out by direct counts of special groups of bacteria and by the analysis of over 1700 strains of bacteria isolated from the fish. Luminous and agar-digesting bacteria occurred seasonally on fish. Luminous strains occurred mainly in the gut contents. A group of sea-water-loving *Pseudomonas* spp. which seem to require sea water for growth on initial isolation was present on the flatfish in variable numbers throughout the year. A more or less distinct intestinal flora was present in North Sea flatfish in which a homogeneous group of micro-organisms provisionally labelled Gut Group vibrios predominates; this group includes luminous bacteria. The bacterial populations of skin and gills were similar in both the fish studied and were composed principally of Gram-negative rods of the *Pseudomonas* and *Achromobacter* genera. The composition of the bacterial flora of the two flatfish, as calculated from an analysis of strains isolated from sea-water agar plates, was: Lemon sole: *Pseudomonas* 60 %, *Achromobacter* 14 %, *Alcaligenes* 8 %, *Flavobacterium* 5 %, corynebacteria 1 %, cocci 1 %, Gut Group vibrios 9 %, miscellaneous 2 %. Skate: *Pseudomonas* 53 %, *Achromobacter* 13 %, *Alcaligenes* 6 %, *Flavobacterium* 9 %, corynebacteria 2 %, cocci 3 %, Gut Group vibrios 12 %, miscellaneous 2 %.

Representatives of most of the known bacterial genera have been isolated from marine sources (ZoBell, 1946). As is to be expected, in view of the low nutrient content of unpolluted sea water, the largest populations of bacteria in the oceans are found associated with solid surfaces such as the sea bed and the bodies of marine animals and plants where the concentration of organic nutrients is relatively high (Russel, 1893; Harrison, Perry & Smith, 1926; Waksman, 1934; Wood, 1953). In particular, large numbers of bacteria occur on the exposed surfaces and in the gut of fish (Lumley, Piqué & Reay, 1929).

It was early recognized that the spoilage of fish as a foodstuff is due almost entirely to the activities of bacteria (Anderson, 1907), and in particular to bacteria of the species normally occurring on fresh fish (Schönberg, 1930). Consequently, several investigations into the bacteriology of commercially important species of fish have been carried out in the last thirty years (see Shewan, 1949; Tarr, 1954). The results of most of these investigations indicate that the bacterial flora of living or freshly caught fish is primarily made up of Gram-negative bacteria, mainly belonging to the genera *Pseudomonas*, *Achromobacter* and *Flavobacterium*. However, some authors (Wood, 1940; Dyer, 1947) have reported that micrococci constituted a large proportion of the types present on the fish they sampled.

There is available therefore a good deal of information, albeit sometimes conflicting, about the nature of the bacterial flora of some types of fish. Little or nothing, however, has been reported concerning the bacterial populations

growing on flatfish, though a few authors have described one or two bacterial strains isolated from individual flatfish (Gibbons, 1934; Thjøtta & Sømme, 1943). The results described in this paper were obtained from an investigation of the bacterial flora of two types of flatfish, skate (*Raja* spp.) and lemon sole (*Pleuronectes microcephalus*). The investigation was both qualitative and quantitative; much of the quantitative data obtained has been published in an earlier paper (Liston, 1956).

METHODS

From October 1952 until December 1954 viable counts were carried out several times a month, using the pour plate method, on samples aseptically excised from the skin, gills and gut contents of freshly caught fish transported to the laboratory in closed sterile tins. The fish were often still alive on arrival at the laboratory and had to be killed before sampling; so the bacteria derived from them can be considered to be representative of the flora of living fish. Counts were carried out in duplicate by using the sea-water agar medium (SWA) and the tap-water agar medium (HHA) described in the earlier paper (Liston, 1956); plates were incubated at 0°, 20° and 37°. From March to December during 1954, plates incorporating *c.* 3 units of penicillin/ml. were additionally included for the counts of skin and gill samples.

Qualitative counts were carried out directly by enumerating the number of luminous and agar-digesting colonies on the normal count plates and, less directly, by comparing the number of colonies appearing on the plates containing penicillin with the number appearing on corresponding plates without penicillin. The luminous colonies were counted in a dark room. The enumeration of the agar-digesting colonies was facilitated by flooding the plates with iodine solution which stains the agar medium except in the area surrounding agar-digesting colonies which are thus readily recognized.

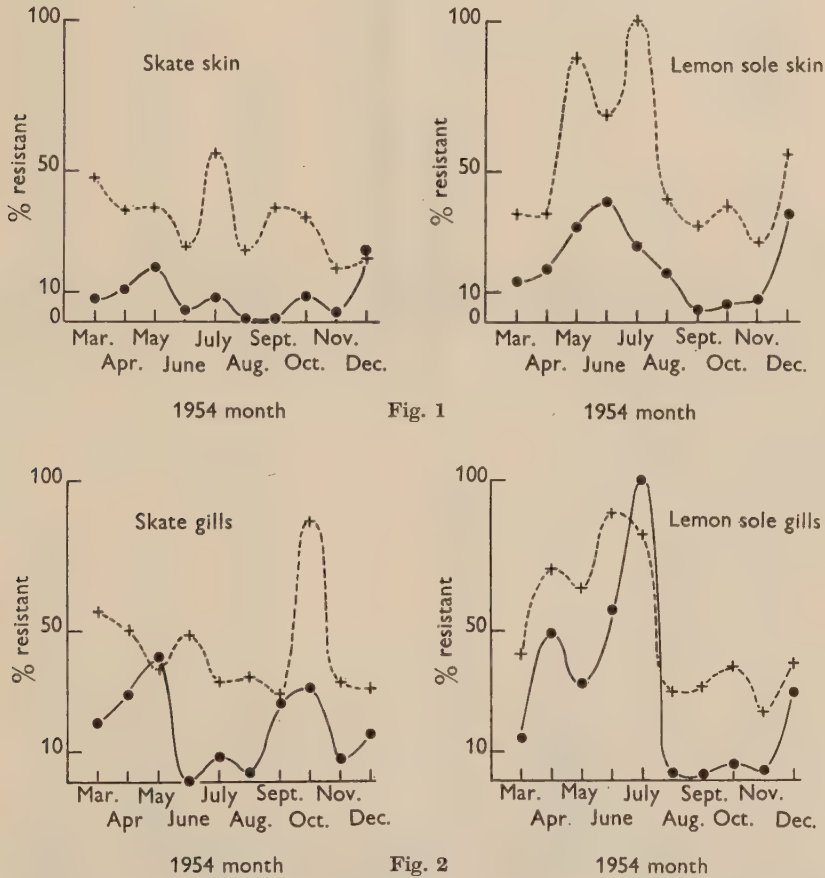
During the first year of the investigation representative colonies were picked directly from the count plates into tap-water or sea-water broth, depending on whether they were derived from HHA or SWA media. In selecting the colonies an attempt was made to ensure that the preponderant types were fairly represented numerically among the isolates, according to their occurrence on the plates. However, in addition, colonies of unusual appearance (e.g. agar-digesting colonies) which did not represent any significantly large part of the flora were also selected so that some estimate of the range of bacterial types occurring on fish could be obtained. The media into which the colonies were picked were incubated at 20° for 3 days in the case of those derived from count plates incubated at 20° and 37° and at 0° for *c.* 14 days when derived from 0° plates. The cultures were then plated out on sea water or nutrient agar to determine their purity (20° and 0° incubation temperatures).

Pure cultures obtained from these plates were examined for morphology and their growth characteristics on solid and in liquid media determined. They were tested (at 20°) for ability to liquefy gelatin, reduce nitrate, alter litmus milk and produce acid from glucose, lactose, sucrose, mannitol, maltose and starch. Most of the cultures were also tested for sensitivity to penicillin,

chloramphenicol, streptomycin, oxytetracycline, and the vibriostatic compound 0/129 (2:4-diamino 6:7-di-isopropylpteridine) in accordance with the method outlined by Shewan, Hodgkiss & Liston (1954) for the rapid differentiation of non-pathogenic asporogenous rods.

RESULTS

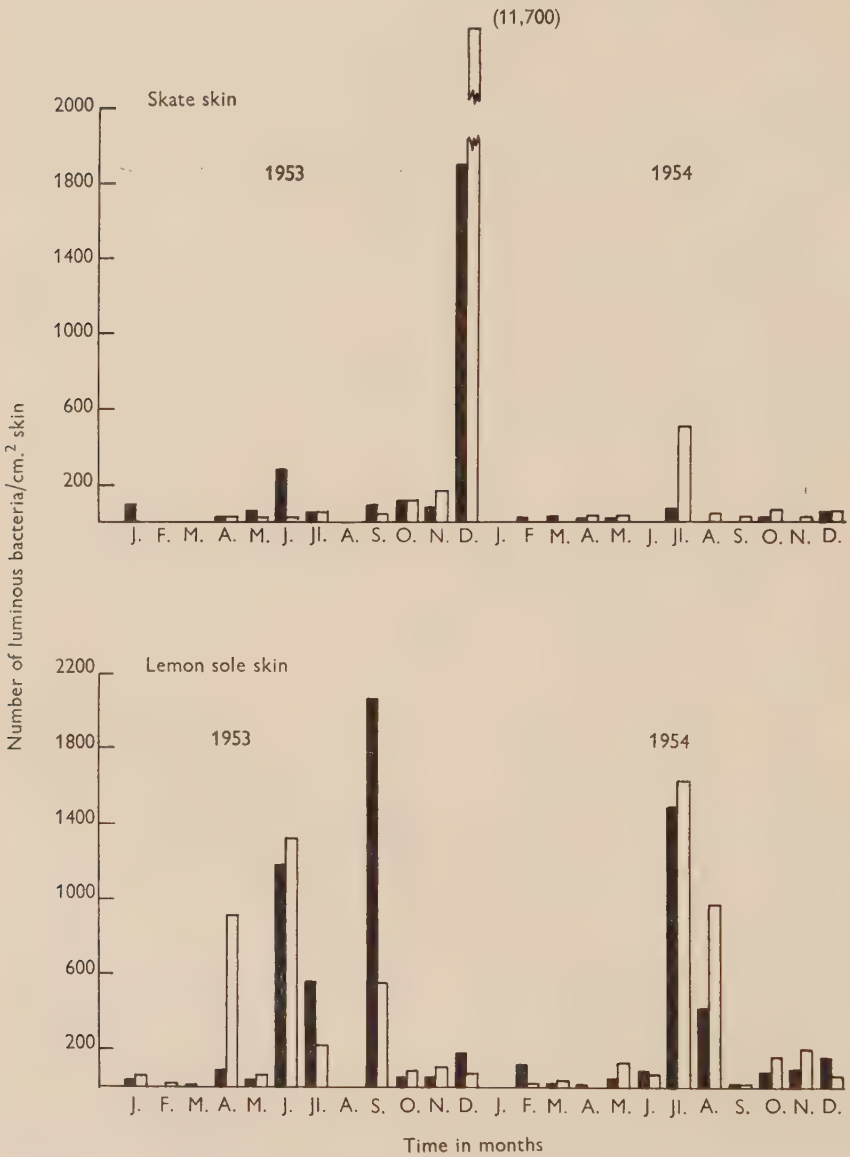
Penicillin-resistant bacteria constituted a variable proportion of the bacterial flora of skate and lemon sole skin and gills during 1954. This is apparent from Figs. 1 and 2 where the penicillin-resistant count is shown as a percentage of



Figs. 1, 2. Percentage of the bacterial flora resistant to 3 units penicillin/ml., during 1954. Fig. 1. Skin flora. Fig. 2. Gills flora. Broken line, counted on SWA medium. Unbroken line, counted on HHA medium.

the corresponding total viable count at 20°. In the case of lemon sole the penicillin-resistant bacteria, on both skin and gills, constituted a relatively large proportion of the organisms present in summer and a relatively small proportion of those occurring in winter. No distinct pattern of occurrence of

penicillin-resistant organisms is apparent for skate skin, but it is noteworthy that they never constituted more than 50 % of the bacterial flora at any time. On the gills of skate there appears to have been a fluctuation in the repre-

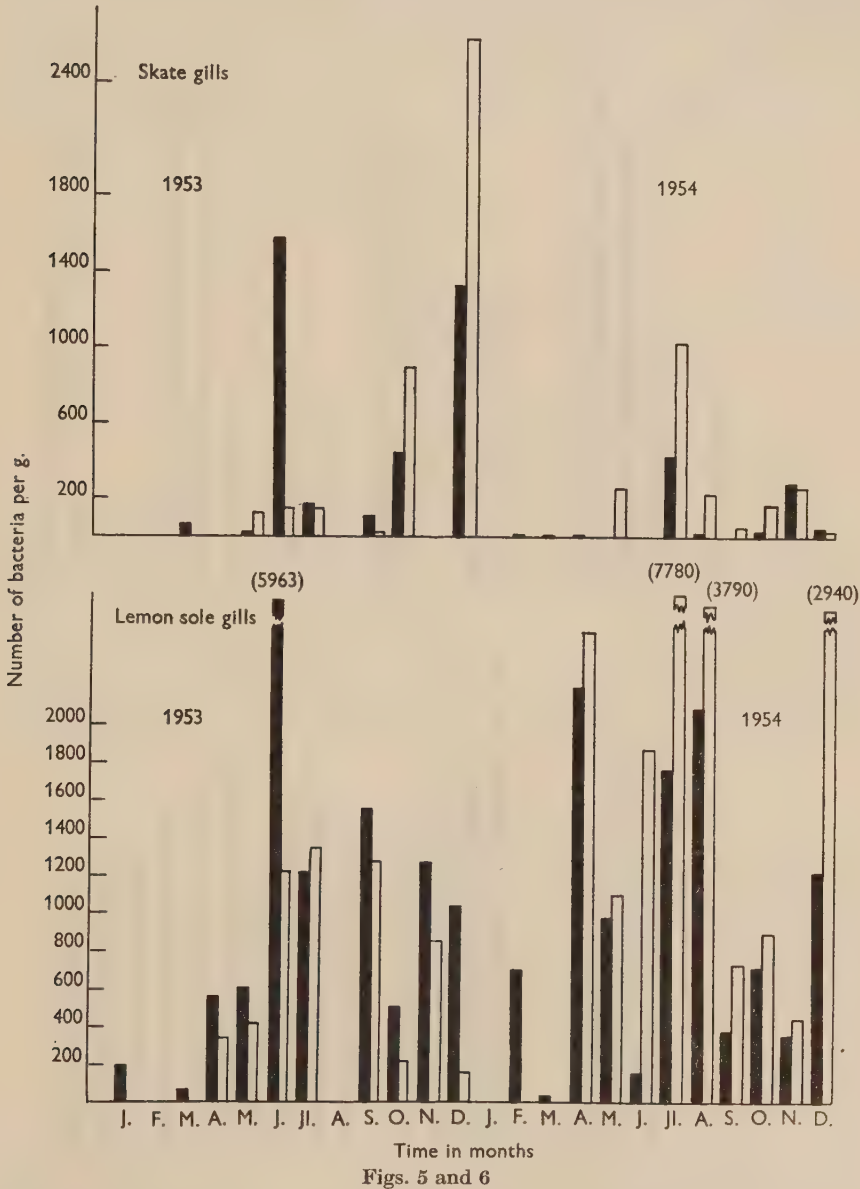


Figs. 3 and 4. For legend see Figs. 5 and 6.

sentation of penicillin-resistant bacteria from a relatively high proportion in spring, through a period of low incidence in summer to a high proportion again in the autumn. While the curves derived from results obtained using SWA and HHA media are similar in shape they are not co-incident and the values

obtained using SWA medium are in general higher than those obtained using HHA medium.

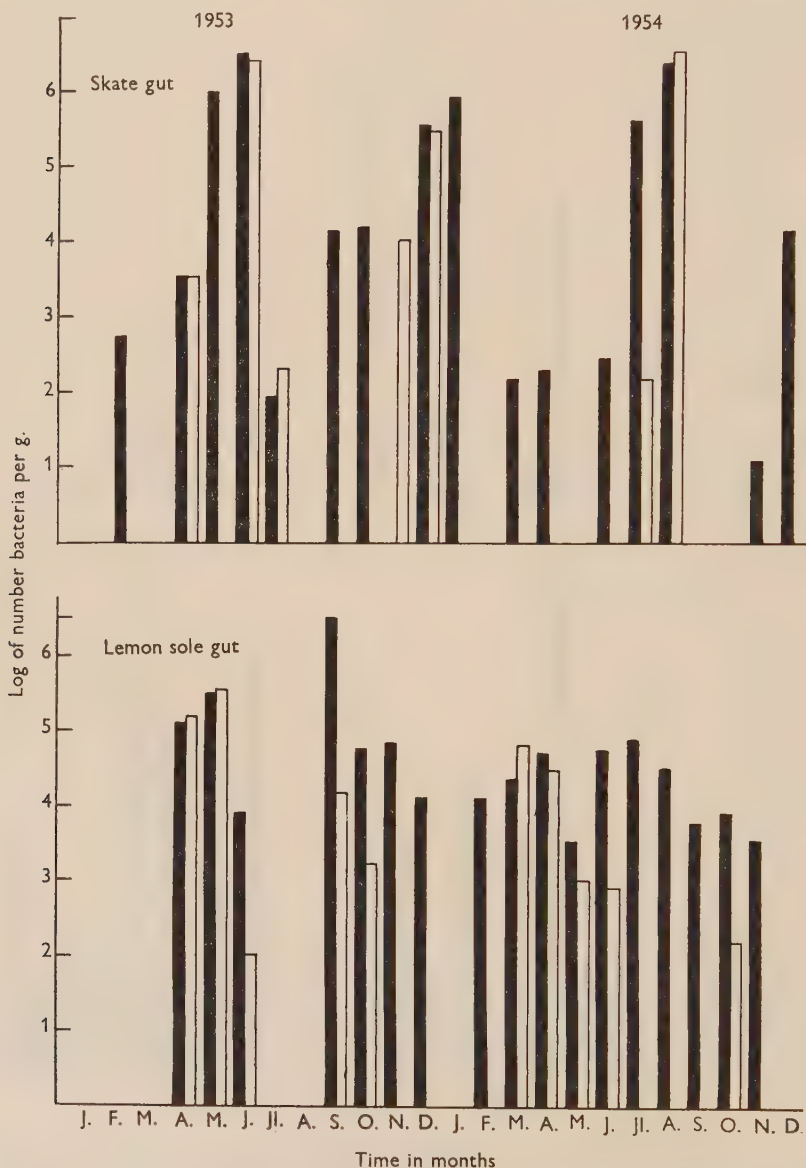
The incidence of luminous bacteria among the organisms appearing on 20° count plates is shown in Figs. 3-9. It has been necessary, because of the large numbers of luminous bacteria often present in gut samples, to express the



Figs. 3-6. Occurrence of luminous bacteria on flatfish during the period January 1953 (J) to December 1954 (D). Fig. 3. On Skate skin. Fig. 4. On Lemon sole skin. Fig. 5. On Skate gills. Fig. 6. On Lemon sole gills. ■, Counts in HHA medium; □, counts in SWA medium.

results of gut counts as log. count/g. From these block diagrams it can be seen that luminous bacteria were more common on the gills than on the skin, in both fish, and on lemon sole than on skate, comparing fish with fish. Very large numbers of luminous bacteria may occur in the gut contents of both fish.

Agar-digesting bacteria were encountered only in very small numbers and never exceeded 1 % of the total bacterial flora appearing at 20° or 0°. They



Figs. 7, 8. Occurrence of luminous bacteria in flatfish gut during the period January 1953 (J) to December 1954 (D). Fig. 7. Skate gut. Fig. 8. Lemon sole gut. ■, counts in HHA; □, counts in SWA.

appeared most frequently on the skin and gills of both fish in the summer and towards the end of the year, but were virtually absent in spring and autumn.

More than 1500 colonies were picked off count plates incubated at 0° and 20°; 212 strains were also isolated from 37° counts. However, the counts at 37° rarely exceeded 1 % of the counts at 20° and therefore the 37° isolates were not investigated in great detail as they were not considered to represent a significant portion of the total bacterial population of the fish examined. Sufficient data have been collected about the organisms isolated at 37° to determine the overall distribution of these bacterial types between the two fish (Table 1). In both skate and lemon sole cocci and *Bacillus* types constitute the bulk of the flora growing at 37°.

Table 1. *Distribution of organisms isolated at 37° from skate and lemon sole*

	Cocci (%)	Bacilli (%)	Gram- negative rods* (%)	Coryne- bacteria† (%)	Yeasts and moulds (%)
Skate	51	26	13	6	4
Lemon sole	56	24	16	4	0

* Mainly pseudomonads and Enterobacteriaceae.

† *Corynebacterium sensu lato* as defined by Jensen (1952).

This distribution between the two fish, according to genera of the bacteria growing at 0° and 20°, as determined by analysis of the characteristics of bacteria isolated from SWA and HHA media is represented in Table 2. The most important features of this distribution are the overwhelming preponderance of Gram-negative rods in the flora of both fish, the apparent reversal of the relative importance of the groups *Pseudomonas* and *Achromobacter* among the organisms derived from HHA and SWA media, respectively, and the similarity between the groups present on the two fish.

Table 2. *Distribution of organisms isolated at 0° and 20° from skate and lemon sole*

	Skate: isolated from		Lemon sole: isolated from	
	SWA medium	HHA medium	SWA medium	HHA medium
	Percentage			
<i>Pseudomonas</i>	52.5	21.7	60.8	19.4
<i>Achromobacter</i>	13.4	24.4	14.1	25.0
<i>Alcaligenes</i>	5.4	14.4	8.2	18.1
<i>Flavobacteria</i>	9.4	3.8	5.0	5.2
<i>Corynebacteria</i>	1.5	4.2	0.9	4.3
Cocci	3.4	1.9	1.4	4.3
<i>G. G. vibrios</i>	12.5	26.9	8.7	22.0
Miscellaneous	1.9	3.2	1.4	1.7

Again in Table 3 the generic distribution of these 0° and 20° isolates is presented according to their original sites of growth on the two fish. From

Table 3 it is apparent that most of the so-called Gut Group vibrios (G.G. vibrios) were derived from gut-samples and were not present in significant numbers in samples from other parts of the fish. The luminous organisms belong to this group of vibrios and their occurrence in very large numbers in the gut has been noted already (Figs. 7, 8). The apparent incidence of G. G. vibrios in gut samples deduced from the analysis of isolates, high as it is, is in fact lower than the actual incidence. Direct observations of count plates during the course of the investigation revealed that these organisms frequently

Table 3. *Distribution by genera of organisms isolated at 0° and 20° according to sites of isolation from the fish*

Key to genera: Ps. = *Pseudomonas*; A = *Achromobacter*; Al. = *Alcaligenes*; C. = *Corynebacteria*; F. = *Flavobacteria*; G.G.V. = Gut Group vibrios; M. = miscellaneous.

Medium														
Sea-water agar							Tap-water agar							
Genera														
Ps.	A.	Al.	C.	F.	G.G.V.	M.	Ps.	A.	Al.	C.	F.	G.G.V.	M.	
Percentage														
Skate														
Skin	63.0	8.6	6.4	5.4	10.2	—	6.4	22.3	38.3	17.5	2.1	4.3	4.2	11.3
Gut	26.7	12.2	2.4	1.6	4.9	48.0	4.2	10.9	3.0	3.0	3.0	1.8	74.0	4.3
Gills	59.5	13.7	6.9	4.0	11.3	—	4.6	24.7	30.6	21.8	2.9	5.3	7.6	7.1
Lemon sole														
Skin	57.0	16.7	9.5	—	9.5	—	7.3	20.2	30.9	22.3	1.6	9.1	5.3	10.6
Gut	34.6	7.7	1.9	5.8	9.6	34.6	5.8	6.1	7.6	7.6	13.6	1.5	59.1	4.5
Gills	62.2	14.5	10.0	—	11.1	1.1	1.1	31.8	31.8	16.5	1.2	3.5	9.4	5.8

constituted nearly 100 % of the bacteria in the gut. The discrepancy between the two results is due to the method of selection of colonies from plates which, as described above, was partly directed towards obtaining examples of the total range of types occurring on fish. Colonies of organisms other than the G.G. vibrios were thus purposely selected from gut sample plates.

The constitution of the bacterial populations of skin and gills is similar not only for each kind of fish but also from fish to fish. Furthermore, the effect of medium of isolation upon the apparent relative proportions of *Achromobacter* and *Pseudomonas* types, noted in the data listed in Table 2, can be seen from Table 3 to be exerted without reference to the origin of the strain—the picture in the case of the gut flora is complicated by the factors of selection already noted.

DISCUSSION

The results obtained in this investigation are in agreement with the hypothesis that the bacterial flora of fish, like the flora of the sea (ZoBell, 1946), is composed principally of Gram-negative rods. No evidence of a preponderance of cocci was noted at any time, though if the organisms isolated at 37° are alone considered, cocci do appear to predominate. This finding is, however, of little

importance since the 37° count is negligible as compared with the 20° count. Nevertheless, the findings of other workers who have reported significantly high proportions of cocci in the bacterial flora of fish cannot be ignored (Stewart, 1932; Wood, 1940; Dyer, 1947). In most of their experiments incubation temperatures of c. 20° were used so that the selective effect of temperature cannot be responsible for the results. Recent investigations concerning the bacterial populations of cod, caught in the same area as the flatfish which were used in this investigation, has revealed that Gram-negative rods again predominate (Mr D. Georgala; personal communication). Consequently, the effect of species of fish on the associated bacterial flora may also be discounted as a cause of the high occurrence of cocci reported by these authors. The most likely reason for their findings seems to be the effect of environment and this is supported by the observations of Wood (1953). Wood noted that organisms isolated in warm Australian waters usually grew well at 37°, while the common finding in the case of bacteria isolated from the cold Northern seas is that they rarely grow at temperatures above 30°.

The relative proportions of the various types of bacteria occurring on flatfish do not remain constant throughout the year. Insufficient information was obtained to determine whether the fluctuations in numbers of penicillin-resistant bacteria occurring on fish is a regular seasonal phenomenon. However, such seems to be the case for the luminous and agar-digesting bacteria, which appear in their greatest number at approximately the same times each year. It is possible that these seasonal variations represent annual fluctuations in the bacterial populations of the sea in which the fish live. Beijerinck (1915) reported an accumulation of luminous bacteria in the North Sea in August and September. Allowing for limited variations of timing from year to year, this period approximately coincides with one of the periods of high incidence of luminous bacteria on flatfish.

The overwhelming preponderance of organisms of the *Pseudomonas* and *Achromobacter* groups in the bacterial flora of flatfish was recognized from a preliminary investigation of strains isolated during the first year of the investigations, as was also the apparent difference in the proportion of the two groups among the bacteria appearing on SWA and HHA media. In general also it was found that, counts on SWA medium were higher than the corresponding counts on HHA medium (Liston, 1956). Since *Pseudomonas* spp. are generally insensitive to penicillin while *Achromobacter* species are sensitive (Shewan *et al.* 1954) it was decided to include plates containing penicillin in the count pattern to obtain some information about the distribution of penicillin-resistant (mainly *Pseudomonas* spp.) and sensitive (mainly *Achromobacter* spp.) organisms occurring on count plates. It appears from the results obtained by this method that the additional colonies which grew on SWA plates were those of *Pseudomonas* spp. which, initially unable to develop on HHA medium, are responsible for the increased proportion of *Pseudomonas* types in the flora derived from such plates. These *Pseudomonas* spp. are obviously sea-water-exacting or at least sea-water-loving so far as primary growth on defined media is concerned; they thus belong to the group of true marine bacteria as

defined by ZoBell (1946). In this respect, however, it should be noted that all strains isolated initially on SWA medium eventually proved able to grow on tap-water media, though some required to be carried through several sub-cultures on sea-water-based media before they would do so. That none of the *Achromobacter* types was inhibited by sea-water agar was proved by the ready growth of all strains on this medium and the isolation of all the types from both media.

Some investigators have reported a distribution of the Gram-negative rods occurring on fish, in which the *Achromobacter* types represent an even greater proportion than appears in Table 2 (HHA columns; see Shewan, 1949). This is undoubtedly due to a great extent to the changing criteria of classification. Reference to the published descriptions of organisms isolated by earlier workers from fish (e.g. Stewart, 1932) shows that many strains which they classified as *Achromobacter* would now be classified as *Pseudomonas* in accordance with the definitive description of this group published in the 6th edition of *Bergey's Manual* (1948). This is particularly true of the proteolytic non-pigmented *Pseudomonas* spp. which are extremely common on fish and which, because of their failure to produce pigment, were usually classified as *Achromobacter* in the past. The antibiotic sensitivity test of Shewan *et al.* (1954) has proved very useful as a method for quickly identifying these organisms.

The third largest group, after the *Pseudomonas* and *Achromobacter* groups, was that of the Gut Group vibrios, so called because they were consistently isolated from gut sample count plates and only occasionally from other sources; a brief description of this group has been published already (Liston, 1954). It should be noted that the isolation of smaller numbers of this type of organism from SWA plates than from HHA plates is due, not to any inhibitory effect of sea water, but to the overall nutritional inadequacy of SWA medium. The organisms seem to be nutritionally exacting and will grow well in a sea-water based medium containing 1.0 % peptone and 0.5 % Lab Lemco. Prominent among the organisms in this group, but constituting only a minority of it, is the luminous group.

Alcaligenes types from fish seem to have been classified simply as *Achromobacter* spp. by earlier workers. However the types isolated in this investigation constituted a distinct and very homogeneous group.

The corynebacteria occurring on flatfish have been identified as *Corynebacterium* spp. *sensu lato* as defined by Jensen (1952). It is only within recent years that corynebacteria have been reported to occur on fish (Wood, 1940; Shewan & Hodgkiss, 1952) but, according to their published descriptions, some strains isolated from fish by earlier workers and described by them as *Flavobacterium* spp. would now be classified as *Corynebacterium* spp. *sensu lato*.

The relatively small number of cocci isolated at 0° and 20° were mainly of the *Micrococcus-Sarcina* type and were, in general, similar to cocci obtained from fish by earlier workers.

The generic distribution of bacteria in the populations of skin and gills was similar in both fish but there were small differences which probably reflect the

varying conditions of the different growth loci on the two fish. Between the bacterial flora of the gut and those of skin and gills, however, there was a very marked difference. It has been repeatedly reported that the gut contents in fasting fish (of many different species) are sterile (see Margolis, 1953) and this finding has been confirmed in the present investigation. Consequently, the bacterial flora of the intestine of fish should depend on the bacteria present in the food ingested by the animal. However, the constant occurrence of Gut Group vibrios as the predominating organism in the gut contents of skate and lemon sole, which consume a fairly mixed diet of small fish, crustaceans and worms of various kinds, implies that the bacterial population of the fish gut does not arise from simple mechanical transfer of organisms from the food. Obviously the conditions in flatfish gut are such as to exert a selective effect on the bacteria ingested and on the evidence of this investigation only the Gut Group vibrios are able to survive in large numbers. It is interesting to speculate on whether these organisms are in this respect analogous to the coliform population of the mammalian gut, though of course the phenomenon of long fasts of several months duration which is normal in the case of fish, does not occur in most mammals.

That a more or less typical commensal bacterial flora occurs on the skin and gills of flatfish is suggested by the repeated isolation of the same type of organism from these areas on fish caught at different times of the year. However, the diverse nature of these bacterial populations of skin and gills is probably at least partly due to the ease of access of these sites of growth from the surrounding water and the generally favourable conditions of growth provided by the slime secreted continuously by fish.

As two lists have been obtained for both of the fish examined, purporting to describe the constitution of their bacterial flora and corresponding to the two media used for isolating individual micro-organisms, it is necessary to decide which list most accurately represents the true state of affairs on the living fish. Since SWA medium has been shown to allow the development of the greatest number of colonies from fish samples and since (excepting the Gut Group vibrios) all isolated strains, whether initially derived from SWA or HHA media, grow readily on SWA medium, the lists obtained from an analysis of organisms isolated on this medium are probably the more accurate. However, the special case of the Gut Group vibrios must be borne in mind, and it is probable that in any future investigation an even better estimate of the nature of the bacterial flora of fish would be obtained by using a sea-water based medium containing larger amounts of peptone and Lemco than the SWA medium used in this investigation.

I wish to thank Mr D. Georgala for supplying me with information on the bacterial flora of North Sea cod. The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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The Type A Phages of *Salmonella typhimurium*: Identification by a Standardized Cross- immunity Test

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SUMMARY: The identification of the type A phages of *Salmonella typhimurium* has hitherto been based on antigenic structure, host range, and plaque characters. The discovery of an apparently phage-free strain of *S. typhimurium*, equally sensitive to all these phages, has enabled us to prepare standardized lysogenic bacteria, alike in all respects except in the symbiotic phage (prophage) they carry. With these it has been possible to test cross-immunity with a high degree of accuracy. The immunity pattern of a collection of 598 type A phages has been examined, and subdivisions in the original types have been revealed. Twelve types are now identified. The test is described and the significance of the findings is discussed.

In early papers (Boyd, 1950, 1954) some details were given of a group of heat-resistant bacteriophages, derived from lysogenic strains of *Salmonella typhimurium*, which were designated type A. These were found to be of three antigenic types, of which the first was common, the second less so, and the third rare. The first of these antigenic types was further divided in accordance with the affinity of its members for certain bacterial hosts; some of the phages were able to multiply on the two indicator strains then in use, *S. typhimurium* 1404 and *S. typhimurium* 1411, while the others multiplied only on 1411. Subsequent work has shown that the types characterized in this way are not all homogeneous and that they can be further broken down by cross-immunity tests. This more extensive subdivision has added precision to the method of distinguishing types of *S. typhimurium* by identifying the symbiotic phage they carry (Boyd, Parker & Mair, 1951). In the present paper a description is given of the development and technique of the cross-immunity test and of the results of its application in the classification of these phages.

METHODS

The culture media and methods of manipulation were those described in previous papers (Boyd, 1950, 1954). The importance of testing samples of different batches of the nutrient agar to be used, and, when a good sample is found, the convenience of securing a large supply sufficient for some months' work, is again emphasized. Because the phages under investigation are very heat-resistant—they survive a temperature of 80° for 30 min.—it has been possible to dispense with filtration and to destroy bacteria and any co-existent B phages in phage preparations by heating to 70° for 30 min.

Lysogenic bacteria and type A phages. The 598 type A phages used in this investigation were recovered from 1400 cultures of *Salmonella typhimurium*.

The majority of these, when received in this laboratory, were recently isolated from infected material—chiefly from the faeces of cases of human infection, but a few from contaminated foods and other sources. 1020 cultures were sent to us by Dr M. T. Parker, and they comprise the majority of the strains isolated in the Manchester area between January 1950 and December 1954. Most of the remainder were from hospital and public health laboratories in different parts of the country, a very few being from overseas.

Indicator strain. An innovation of great importance was the use, in the later stages of the investigation, of the recently acquired indicator strain, *Salmonella typhimurium* Q1 (Boyd, 1956). As far as can be ascertained, Q1 contains no symbiotic phage: it is sensitive to all the known phages of *S. typhimurium*, of both A and B types. The A phages all multiply well on it and, under suitable conditions, produce a lysate containing a high concentration of free particles. Further, when the appropriate technique is used, Q1 can be 'symbiotically' infected with all these phages, and so rendered lysogenic. In this way a series of lysogenic cultures has been prepared, in all of which the bacterium is the same, and only the symbiotic phage (prophage) which they carry is different.

Preparation of phage suspensions and lysogenic strains of Q1. Phage suspensions and lysogenic strains of Q1 are prepared as follows:

(1) Inoculate the lysogenic *Salmonella typhimurium* (usually a subculture of the original strain recovered from infected material) into broth, and incubate overnight.

(2) Spin until clear: the supernatant fluid contains the free phage particles liberated from the lysogenic bacteria.

(3) Heat to 70° for 30 min. to kill any remaining bacteria and destroy B phages (if present).

(4) Add 0.5 ml. of this supernatant fluid to 4.5 ml. of an actively growing broth culture of Q1 containing approximately 10^8 bacteria/ml. Incubate until clearing occurs, or overnight if there is no clearing.

(5) Spin and heat to 70° for 30 min.

(6) Titrate by making decimal dilutions in broth to 10^{-6} . This can be done with sufficient accuracy by a dropping technique, using standardized pipettes. Flood a plate of nutrient agar with a well-grown culture of Q1, pipette off the excess, and allow the plate to dry. Place a loopful of each dilution of phage on a marked spot on the plate. Allow to dry. Incubate overnight.

(7) Examine with a plate microscope to ensure that only one type of A phage is present. In the very exceptional cases where two are present, one is usually less numerous than the other, and its scanty plaques can be seen superimposed on the coalesced plaques of the more common variety.

(8) Read the results of the titration. The plate will show all gradations from solid-centre 'patches' in the low dilutions to discrete plaques in the high. The Critical Test Concentration (CTC) is the highest dilution at which the plaques are completely confluent, so that the patch appears quite homogeneous and has a smooth edge. This point is likely to lie somewhere between two of the original decimal dilutions. If necessary, a further test with intermediate dilutions can be put up, but with experience there is little difficulty in

estimating the correct point by gauging the density of the patch which just fails to show CTC.

(9) For test purposes, dilute the phage preparation to CTC.

(10) With a sterile platinum needle take a small inoculum of bacteria from the centre of the patch given by the 10^{-1} dilution. Suspend this in about 1 ml. of broth, and immediately plate from this on to nutrient agar. Incubate.

(11) Next morning the plate will show some colonies with markings which indicate phage contamination, and others which appear normal. Select one of the latter—they are almost without exception lysogenic—and inoculate into broth. Incubate.

(12) When this culture shows slight opalescence, sub-inoculate into a growing broth culture of Q1. After 3–4 hr. incubation, place a drop of this second culture on nutrient agar sown with Q1, and incubate overnight. The development of plaques or a patch will confirm that the culture is lysogenic.

(13) Plate from the broth culture made in (11), and incubate. A uniform crop of colonies of normal appearance, completely devoid of phage markings, confirms that it is uncontaminated by extraneous free phage.

(14) Make stock cultures from the colonies on this plate.

The points of fundamental importance in this somewhat elaborate technique are (a) the phage particles from the original lysogenic strain at no time pass through any other host organism except Q1, and (b) the lysogenic strains of Q1 are identical except for the prophage they carry.

RESULTS

Preliminary observations on cross-immunity

We were first made aware of the existence of more than one type of A1 phage by the chance observation that a freshly isolated temperate phage, which had been provisionally identified as A1, produced typical patches and plaques when 'spotted' on a plate sown with a lysogenic strain carrying A1. As a lysogenic bacterium is 'immune' to the phage it produces, this new phage was clearly of a different type from that carried by the organism on which it was 'spotted'. Following this observation, a series of cross-immunity tests was carried out with all the phages in our collection which had been identified as A1. The results showed that they were of at least three different types. However, when lysogenic bacteria carrying these three types were exposed to the action of the other A phages, the results were not uniform, and the position was somewhat confused. The lysogenic bacteria used in these tests were sub-cultures of those isolated from faeces or other infected material.

Similar tests were then carried out on a cross-section of the many strains belonging to the type originally designated A2. (At this time the types now placed as A2e and A2f had been identified but were classified separately.) The strains in this first sampling fell into three clear-cut types, but a few strains were found, when the complete collection was tested, which did not exactly match any of these types, and certain anomalous results, comparable to those seen in the A1 series, were also encountered.

Among the different factors capable of causing the minor irregularities in the reactions of the A1 and to a lesser extent the A2 types, it seemed possible that the host bacterium might play a part. Thus, there might be some difference in the receptor mechanism which is involved in the adsorption of the phage particles, or the bacterium might carry another not very obvious prophage which had nevertheless antagonistic or immunizing properties. Some support is given to this theory by the differing sensitivity of 1404 and 1411 to these A phages, neither of these indicators being capable of supporting all of them equally, though they all will grow to a greater or lesser extent on 1411. It is of interest to record that nothing has been discovered to explain the differing sensitivity of 1404 and 1411 to the A phages. Whether or not they carry a weak B prophage—this must be regarded as a possibility—neither carries an A prophage which could confer immunity. No difference has been found in their biochemical reactions, and Dr Joan Taylor reports that they are of identical antigenic structure. With the discovery of Q1, which is equally sensitive to all the A phages, it became possible to by-pass the problem of variations attributable to the host bacterium. Using standardized lysogenic strains of Q1 prepared in the way that has been described and standardized suspensions of the temperate phages, the irregularities in the cross-immunity test disappeared.

The extent to which the different phages act on sensitive lysogenic bacteria is variable. Some appear much more active than others, and produce patches and plaques which are not to be distinguished, either in appearance or in number, from those they produce on phage-free Q1. Others produce shallow, weak plaques which are most easily seen by transmitted light, and which are present in greatly reduced numbers in comparison with the yield given on the indicator strain. Between these extremes are intermediate forms of varying activity.

Some of the strong lysates containing a high concentration of temperate phage particles exercise a lytic action on certain of the lysogenic bacterial strains. The lytic agent concerned is non-particulate, and its action fades out as the phage preparation is diluted, in most cases having disappeared when a dilution of 10^{-2} is reached. When present, the lysis tends to obscure the action of the weaker phages, and it has therefore been found advantageous to dilute phages which are to be tested for cross-immunity beyond the point where lytic action occurs. This is achieved, and at the same time the necessary degree of standardization is introduced, by diluting the phage to the Critical Test Concentration (CTC) of Craigie & Yen (1938), as already described.

Technique of the cross-immunity test

The technique of the cross-immunity test is simple. A loopful of a broth culture of each of the lysogenic strains is spread over an area about an inch in diameter on the surface of nutrient agar in a Petri dish and allowed to dry, the back of the plate being marked so that the different cultures can be identified. On the centre of each inoculated area there is placed a standard loopful of the phage which is being tested, diluted to CTC; this in turn is allowed to dry, the plate incubated overnight, and the results read next day. The reaction may

range from a solid patch through decreasing numbers of separate plaques to a negative result. Symbols ranging from + + + + to + and - are used to score the results. While phages of the same type may show some variation in the intensity of their action, attributable possibly to the adjustment of CTC, the overall pattern of reactions is constant.

The cross-immunity pattern

The compiled results of the cross-immunity test are set out in Table 1, in which for convenient reference the antigenic relationships and host range of the different types are also shown.

Twelve types have been differentiated. The original A1 and A2 types are each subdivided into four. A5 (Boyd, 1954) is now shown as A2e, as it has the same antigen and host range as the other A2 types. A sixth variety, which had been recognized as a separate type, but has not so far been described, is included as A2f. All strains of A3 appear to be identical. The position of A4 will be given in detail later.

Of the 598 strains of phage examined, 592 fall into these 12 types. The remaining 6 give anomalous results.

Ten of the 12 types (Table 1) have a distinctive cross-immunity pattern by which they can be readily identified. While some variations in the degree of the positive reactions may occur, this pattern is constant. Two of the phages (A2f and A3) do not react with any of the lysogenic strains—or, to put it the other way round, all the lysogenic strains are immune to these two phages. Though they cannot be directly identified by cross-immunity, they are readily distinguished by antigenic pattern, host range, and plaque characters.

It will be noted that the test can be performed in two ways, either by testing the unknown phage against the known lysogenic bacteria—the method which has been described—or, vice versa, by rendering Q1 lysogenic with the unknown phage and testing its immunity to known phages. Either method is equally reliable, but the former is preferred because it is both simpler and quicker to carry out, a standardized phage being easier to prepare than a lysogenic strain of Q1.

Plaque characters of different types

The characters of the plaque may be of some assistance in confirming the identity of a phage but they are apt to show considerable variation and must be interpreted with caution. The quality of the nutrient agar used, differences in the thickness of the agar layer (even the relatively minor differences resulting from a slightly convex or concave bottom to the Petri dish), the concentration of bacteria in the indicator culture with which the plate is sown—these and other factors influence the development of the phage and make it difficult to define the normal plaque. The typical plaque formed by all A phages appears as a saucer-shaped depression in the sheet of bacterial growth formed by the indicator strain. A little opaque dome-shaped mound of bacteria, like a small colony, occupies the centre of the saucer. Immediately surrounding this there is a narrow rim which may be comparatively clear, and which merges into

Table 1. Results of cross-immunity test; also showing antigenic relationships and host ranges

Type of phage	Anti-genic pattern	Host range		<i>Salmonella typhimurium</i> Q 1 carrying prophage											
		Indicator strain	1404 1411	A 1a	A 1b	A 1c	A 1d	A 2a	A 2b	A 2c	A 2d	A 2e	A 2f	A 3	A 4
A 1a	Alike	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A 1b		+	+	+	+	+	+	+	+	+	+	+	+	+	+
A 1c		+	+	+	+	+	+	+	+	+	+	+	+	+	+
A 1d		+	+	+	+	+	+	+	+	+	+	+	+	+	+
A 2a		-	+	+	+	+	+	+	+	+	+	+	+	+	+
A 2b		-	+	+	+	+	+	+	+	+	+	+	+	+	+
A 2c		-	+	+	+	+	+	+	+	+	+	+	+	+	+
A 2d		-	+	+	+	+	+	+	+	+	+	+	+	+	+
A 2e		-	+	+	+	+	+	+	+	+	+	+	+	+	+
A 2f		-	+	+	+	+	+	+	+	+	+	+	+	+	+
A 3	Distinct	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A 4	Distinct	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Under host range: + = multiplies freely; ± = multiplies badly; - = does not multiply.

In cross-immunity table proper:

+ + + + = confluent or almost confluent plaques when the phage is applied at Critical Test Concentration (CTC);

+ + = semi-confluent plaques at CTC;

+ = numerous discrete plaques at CTC;

± = small numbers of plaques at CTC;

- = may or may not be plaques at CTC; in these cases, higher concentrations of phage produce plaques which may have been diluted out.

a zone of increasing opacity, the inner halo. The inner halo extends to the edge of the saucer and varies in width, depending on the steepness with which the saucer shelves. With certain phages, and certain indicator strains, there is a further partially clear zone—the outer halo—which surrounds the saucer and may be as wide as the plaque.

When Q1 is used as indicator, some of the types have characters which are sufficiently obvious and constant to be of value in confirming identity.

(1) The plaques of A2e and A2f are approximately half the size of the others, A2f being the smaller of the two. (On a good medium, the solid centres of the large-plaque types measure up to 1 mm. across, while the outside measurements of the saucer are from 1.5 to 2 mm.)

(2) A2b has a small rather irregular centre and a wide inner halo which may show concentric rings, giving it the appearance of a target.

(3) A4 has a characteristically granular rim and inner halo.

A2e, A3 and A4 have little or no outer halo, whereas in the other types this feature is well marked.

Incidence of the different phage types

Table 2 shows the numbers of the different phage types identified from 1948 to 1954, and Table 3 the localities in which the lysogenic strains of *Salmonella typhimurium* carrying these phages were isolated.

Table 2. *Isolations of type A phages of Salmonella typhimurium by years*

Phage	Pre 1948	Year							Total
		1948	1949	1950	1951	1952	1953	1954	
A1a	.	.	1	1	1	.	4	16	23
A1b	1	.	2	.	.	1	1	1	6
A1c	3	.	.	.	3
A1d	5	1	.	.	6
Anomalous									
A1	1	1
A2a	.	23	11	50	19	12	100	61	276
A2b	.	.	.	22	89	70	6	.	187
A2c	.	3	3	33	39
A2d	.	.	4	4
A2e	3	.	.	3
A2f	9	9
Anomalous									
A2	.	.	1	4	5
A3	.	1	2	.	.	1	21	5	30
A4	.	.	.	2	3	.	.	1	6
Grand total									598

The figures indicate the number of strains of *Salmonella typhimurium* carrying each phage. With a few exceptions, these were isolated from infected material in the year indicated.

The original A1a, the first of these phages to be identified, was recovered from a specimen of faeces before the investigation recorded in this paper started, and was maintained as a lysogenized strain of 1404. It has since been

recovered repeatedly from cultures of *S. typhimurium*, and is thus a well-established type, though not, in our experience, as common as certain of the A2 types.

Only a few A1b strains have been found, but they have cropped up in different years. One was kindly given to us by Dr B. A. D. Stocker. It is Lilleengen's LT22 strain 409 (Lilleengen, 1948), and was the strain used by Zinder & Lederberg (1952) in their transduction experiments. It was originally isolated in Chile. In Lilleengen's experience this was a common type, providing 80 out of the 667 strains he examined, and being recovered from man, the horse and the hen.

Table 3. *Geographical distribution of lysogenic Salmonella typhimurium strains carrying A phages*

Phage	Localities in which the lysogenic bacteria have been isolated
A1a	Portsmouth, Manchester
A1b	Chile, Liverpool, Manchester
A1c	Manchester
A1d	Manchester
A2a	Norwich, Copenhagen, Winchester, Stafford, Salisbury, Oxford, Liverpool, Portsmouth, Birmingham, Woolwich, Neasden, Ipswich, Manchester
A2b	Portsmouth, Liverpool, Manchester
A2c	Norwich, Liverpool, Manchester
A2d	Liverpool
A2e	Manchester
A2f	Manchester
A3	Wakefield, Manchester
A4	Germany, Manchester

Of the 1400 strains of *Salmonella typhimurium* examined, 1020 were from the Manchester area, and only 380 from elsewhere.

A1c is closely related to A1b, and shows only minor differences in the cross-immunity pattern. The validity of these minor differences is confirmed by the plaque characters as revealed by transmitted light, for A1c has a noticeably granular rim, which A1b lacks. Another difference is that A1c multiplies badly on the indicator strain, 1411. The three strains of A1c came from a common source and represent an isolated incident.

All six strains of A1d came from Manchester between August 1951 and February 1952. It seems probable that the infections from which they were derived were from a common source or were related one to the other.

A2a is the type first described as A2 (Boyd, 1950). It was found early in the investigation and has been one of the commonest types in the Manchester series, predominating in some years and relatively uncommon in others. It has also been found in strains of *Salmonella typhimurium* from other parts of the country, and is without doubt a stable and permanent type.

A2b was not differentiated from A2a until the cross-immunity test was devised, although it had long been suspected that all the types designated A2 were not alike. Fluctuations in its incidence can be seen in Table 2. While the majority of the strains came from Manchester, several have been recovered

from specimens from other parts of the country. As already mentioned, it has distinctive plaque characters.

A culture of *Salmonella typhimurium* carrying A2c was found in a batch of strains kindly sent by Lt.-Col. H. J. Bensted, Central Public Health Laboratory, Colindale, in 1948, and 2 strains came from Norwich in the same year. Three strains were received from Liverpool in 1949. This type did not, however, appear in the Manchester series until 1954, when 29 cultures of *S. typhimurium* carrying this phage were isolated.

The original number of A2d strains was 5: unfortunately 1 was lost before the cross-immunity test was brought into use. All 5 were from cultures from the same outbreak of enteritis, 4 from human infections and 1 from the rabbit-pie which was responsible. When these phages were first examined, they were thought to be of the usual A2 types prevalent at the time, and no particular significance was attached to the incident. The cross-immunity test reveals an unusual source of infection which would not otherwise have been suspected.

The 3 strains of A2e were from the Manchester area and were encountered between July 1952 and February 1953. This is the only occasion on which this type has been found.

A2f was found in 9 strains of *Salmonella typhimurium*, all recovered in the Manchester area in fairly close sequence in October and November 1954. It may safely be assumed that they emanated from a common source.

A3, which has a distinctive antigen, has been found in cultures sent from different places at different times throughout the investigation. All strains have an identical cross-immunity pattern.

A4 has also a distinctive antigen and has plaques of characteristic appearance. Nevertheless, cross-immunity tests divide this small group of 6 strains into 3 types. The first 2 (Boyd, 1950) come from bacteria isolated from human infections in Manchester. The cross-immunity results given in Table 1 are those of this pair. The second 3 strains were isolated from pigeons in Germany. These have a distinctive cross-immunity pattern. The last strain is of considerable interest. It occurs, together with an anomalous A1 phage, in a strain of *Salmonella typhimurium* of human origin which is the only one in the series known to carry two type A phages. No difficulty has been experienced in isolating these phages in pure culture, and there is no valid reason to suggest that there has been any intermingling of the phage characters, though this naturally suggests itself as an explanation of the aberrations in cross-immunity.

Because of the very small numbers of A4 isolated, they have not been shown as separate subtypes. This had best await further isolations.

Little need be said of the 6 phages which do not fit into this classification. One is the A1 phage found in association with A4, which has just been mentioned. Of the 5 A2 group phages, 1 was received from the Central Public Health Laboratory, Colindale, and is of unknown origin. The other 4, of which 2 are alike, came from Manchester in 1954. All may well be isolated representatives of types which are common elsewhere.

DISCUSSION

The results given by the standardized cross-immunity test are definite and consistent. The use of a common non-lysogenic 'host' on which temperate type A phages can be grafted to produce lysogenic strains, or, alternatively, can be propagated to produce high concentrations of free phage particles, has eliminated extraneous factors which might interfere with the accuracy of the test. The repeated isolations of phages which give identical reactions, in some cases at intervals of years, is good evidence both of the reliability of the test and of the validity of these types.

The frequency with which the different types have been recovered (i.e. the total number of strains of each type) is governed by epidemiological factors and does not necessarily have any other significance. Most of the types of which only small numbers have been found (e.g. A1c, A1d, A2d, A2e, A2f, A4) were recovered over a short period of time, each from a series of cases likely to have been related, whereas the more common types (A1a, A2a, A2b, A2c, A3 and, to a lesser extent, A1b) have recurred in different places throughout the years.

As human infection with *Salmonella typhimurium* is usually food-borne, it is possible, if not indeed probable, that the rarer types have been introduced—perhaps in foodstuffs such as dried eggs—in a strain of *S. typhimurium* from an area where this particular type is prevalent. If the resultant outbreak of enteritis in the community is effectually checked, and if none of those infected become carriers, both host-bacterium and phage will automatically disappear. On the other hand, the common and frequently recurring types are likely to be from strains of *S. typhimurium* which have become established in carriers in the areas which have been investigated (e.g. Manchester and district), where fresh outbreaks are caused from time to time by infection disseminated by these carriers. There is no evidence that the pathogenicity of *S. typhimurium* is in any way influenced by its phage content, and there can be little doubt that more extensive sampling over a wider area would reveal the locality in which those types, rare in our investigations, are of common occurrence either in man or in animals which can contaminate human food.

An alternative, but unlikely, explanation of our findings, is that the rare types result from some form of re-combination which takes place when one type of temperate phage multiplies in a bacterium carrying the prophage of another type. *In vitro* experiments, which will be recorded in greater detail in a later paper, have so far failed to reveal changes of type occurring in this way. In these negative experiments the numbers of bacteria and phage particles involved were massive: in natural surroundings, chance encounters between lysogenic bacteria and temperate particles to which they are sensitive must be, in comparison, of the greatest rarity. The possibility of rare types taking origin in this way cannot be ruled out, but so far has not been experimentally proved.

The repeated recovery of the more common types, particularly A1a, A2a, A2b and A3 in different parts of the country and at different times throughout the investigation, reveals the stability of these phage types in the lysogenic

strains of *Salmonella typhimurium* which carry them. This is well illustrated in the case of A2a and A2b. Both of these types commonly occur in association with B2, a coincidence which at first wrongly fostered the belief that all were alike and probably came from the same source. Differences in plaque characters and activity of growth on 1411 were nevertheless observed, and it was at one time thought that some gradual change might be occurring in the character of the phage. This theory was decisively disproved by the cross-immunity test, which revealed two distinct varieties of A2 (A2c came into the picture at a later date), both of which had been present throughout the investigation, one predominating at one time and the other at another. As an interesting addendum nine strains of *S. typhimurium* from the Manchester area were sent for special investigation in 1955: of these, eight were A2a and one was A2c. It is significant that these strains continue to maintain their identity, though they have been occurring in close association with each other in the same locality for a number of years.

Are further varieties of type A likely to be encountered? The answer to this query is definitely in the affirmative. The strains which have been noted as anomalous are, in all probability, types which are common in some other place, and there is no reason to doubt that there are others still to be found. This point can be settled only by widespread sampling and matching the reactions of the phages isolated against the standard pattern.

The wider range of phage types revealed by the cross-immunity test, and the certainty with which they can be identified, adds greatly to the reliability of the method of typing strains of *Salmonella typhimurium* described in a previous paper (Boyd *et al.* 1951). The value of the method will be further enhanced when the B phages are also classified by a cross-immunity test of this kind. This test will not be as easy to carry out with B phages as it has been with A phages, because the former are relatively heat-labile and therefore cannot be prepared in pure sterile culture by the simple process of heating. However, the difficulties are not insuperable, and it is hoped to undertake this work at some future date.

This method of phage-typing strains of *Salmonella typhimurium*, by isolating and identifying the symbiotic phage (prophage) they carry, is of greater accuracy than the modified Craigie method used by Lilleengen (1948) and by Felix (1956), which cannot properly cope with the subtleties introduced by multiple infections with A and B phages. Such multiple infections are of very common occurrence and result in many different and distinctive combinations, each of which breeds true. Nevertheless, the simple technique of the method used by Lilleengen and Felix and its accuracy in matching—as opposed to typing—lysogenic strains of *S. typhimurium*, make it a valuable test for routine investigations of a local nature. Its limitations were recognized by Felix himself, and led him to concur in the use of locally produced typing phages.

From the results shown in Table 1 it would appear that certain phages are much 'stronger' than others. Thus, strains of *Salmonella typhimurium* carrying A1b, A1c, A2d and A2e are resistant to the majority of the A phages,

whereas the free phage which they produce can attack and multiply in many of the lysogenic bacteria of the group. Conversely, others—notably A1a, A2f and A3—confer little immunity on their host bacteria and have little capacity for attacking the other lysogenic bacteria. In another paper (Boyd, 1956) an outline has been given of the way in which these phages react with different lysogenic bacteria. In general, the ‘strong’ phages evict and replace the prophage of lysogenic bacteria carrying the weaker phages: the less strong phages may evict or may produce double infection, and, where this is reciprocal absence of immunity, double infection usually, but not invariably, results. These observations, details of which it is hoped to record in a later paper, have something in common with the findings of Bertani (1954), who worked with *Shigella dysenteriae* and mutants of a phage to which it is sensitive.

We are indebted to many bacteriologists throughout the country, and particularly to Dr M. T. Parker, for sending cultures of *Salmonella typhimurium*, to Dr Joan Taylor for a number of analyses of the antigenic structure of different strains, and to Mr R. A. Miles for much technical assistance in the earlier part of this investigation.

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Viable Counting of *Mycobacterium tuberculosis* in a Silica Gel Medium

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SUMMARY: A method is described in which deep counts of *Mycobacterium tuberculosis* are made in a defined medium solidified with silica gel. The method was accurate, simple and safe. The contamination rate was low. Deep counts were higher than surface counts on plates. The defined medium was superior to Kirschner medium.

The enumeration of viable bacteria is most frequently and accurately carried out on the surface or in the depths of solid medium. A surface viable count method for *Mycobacterium tuberculosis* using oleic acid + albumin agar medium was described and statistically analysed by Fenner (1951). Yegian & Budd (1951) also described a pour plate technique for mycobacteria using the same medium and they suggested that it yielded a higher count than the former method. A disadvantage of the surface count technique is the high rate of contamination, which in our hands has occasionally been as high as 15 % of the plates inoculated. In order to avoid contamination Mitchison (1950) and O'Hea (1955) described surface count methods in which the medium was distributed into small bottles and each of these was inoculated with a drop of the appropriate culture dilution. These methods are inconvenient where accurate work is required because of the large number of bottles used. Hirsch (1954) pointed out that agar has an inhibitory action on the growth of tubercle bacilli, only in part due to binding of the ferric ion. Knox (1955) described a technique for deep counts in a semi-solid medium of low agar concentration, contained in tubes. In this method optimal conditions for growth varied according to the depth of the colonies in the column of the medium and this may possibly be a source of error. Knox has not published accounts of comparisons with Fenner's method. We have investigated the use of a deep culture medium solidified with silica gel in place of agar. The counts can then be set up in narrow-necked bottles which decreases the incidence of contamination and allows the formation of a thin well-aerated layer of medium.

METHODS

Organisms. *Mycobacterium tuberculosis*; strains H37Rv and H37Ra, obtained from Dr R. J. W. Rees (Medical Research Council Laboratories, Mill Hill, London); Branch, a bovine strain, obtained from Dr A. Q. Wells (Sir William Dunn School of Pathology, Oxford); strain B.C.G., Copenhagen strain, obtained from current batches of vaccine; strain B, a variant of H37Rv highly resistant to isoniazid and catalase-negative (Barnett, Bushby & Mitchison, 1953); strains 0139 and 0704, recently isolated from patients with

pulmonary tuberculosis and sensitive to streptomycin, *p*-aminosalicylic acid (PAS) and isoniazid; strains 0942 and 0435, human strains recently isolated from patients with pulmonary tuberculosis and resistant to PAS but sensitive to streptomycin and isoniazid.

Media. Unless otherwise stated, the medium used was the 7H-3 medium described by Middlebrook, Cohn & Schaefer (1954). Since a multiplicity of media was described by these authors, the method of preparation is given:

Basal medium. KH_2PO_4 , 0.2 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.26 g.; sodium-L-glutamate, 0.1 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.1 g.; sodium citrate. $2\text{H}_2\text{O}$, 0.02 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.0001 g.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0002 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0002 g.; pyridoxine hydrochloride, 0.0002 g.; biotin, 0.0001 g.; glycerol, 0.4 ml.; ferrous ammonium citrate, 0.02 g.; distilled water to 100 ml. Adjust pH to 7.2 with HCl. Autoclave at 15 lb./sq.in. (120°) for 20 min.

Oleic acid-albumin complex. (a) Bovine Albumin Fraction V (Armour) 10 g.; glucose, 4 g. Dissolve in 0.85 % saline to 190 ml. (b) Oleic acid, 0.12 ml.; 0.05 N-NaOH, 10 ml. Mix (a) and (b). Seitz filter and add 20 ml. to 100 ml. of basal medium. For plates solidified with agar the 7H-3 basal medium was diluted with 100 ml. distilled water. Malachite green to a final concentration of 0.0001 % and agar to 1.4 % were added. The medium was autoclaved (15 lb./sq.in. for 20 min.) and then 20 ml. of the oleic acid albumin complex added.

A liquid medium modification of the 7H-3 medium was also used, in which glycerol was omitted from the basal medium and 100 ml. water, 0.025 % Tween 80 (Atlas Powder Co.), 0.2 % glucose and 0.5 % bovine albumin were added.

Kirschner Medium (Mackie & McCartney, 1953) was used in one experiment with the following modifications. Penicillin was not added to the horse serum; the pH of the medium used for agar plates was adjusted 6.7 with N-HCl; the medium used with the silica sol was prepared at double concentration and its pH was adjusted to 7.2 with N-HCl.

Silica sol. A 2.6 % sol was prepared according to the method described by Smith (1951), using Zeo-Karb 225 (Permutit Co. Ltd., London, W.4) as the cation-exchange resin. The sol was sterilized by autoclaving at 15 lb./sq.in. for 15 min. and was stored in c. 100 ml. volumes in Pyrex flasks at 4° . It remained usable for at least a week.

Diluent. Sterile distilled water or 0.1 % bovine albumin fraction V in distilled water were used in different experiments.

Use of silica gel solidified medium

The medium to be solidified with silica gel was distributed with aseptic precautions in 3 ml. amounts into 2 oz. sterile medical flats with screw-caps. The bottles were incubated at 37° for 2 days as a sterility check and then could be stored, when necessary, for several months at 4° . Dilutions of cultures or of tissue homogenates were then added, usually in 0.2 ml. volumes from 1 ml. graduated pipettes. These operations were carried out inside an inoculation hood using a Pumpett automatic pipette control (Shandon,

London). Accurate counts were obtained using 40-fold dilution steps. After inoculation of all the bottles on any day, 3.2 ml. of the silica sol were added to each, the contents were mixed and the bottles immediately laid flat on the bench, so that a layer of medium about 3 mm. deep covered one of the larger surfaces. Gelling began within 5 min. and was complete after *c.* 1 hr., during which period the bottles were left undisturbed. They were incubated for 4 weeks at 37° and colonies were then counted as in pour plates. When the number of colonies was between 600 and 2000/bottle an approximate count could be obtained by counting within a series of thin-ruled areas at intervals along the bottle. Less accurate counts up to 10⁵ colonies/bottle could even be made with a plate microscope containing a square of known area in the eye-piece focal plane.

For surface counts on plates the method of Fenner (1951) was used. Dropping pipettes were calibrated both with a micrometer screw gauge and by measuring the volume of a large number of drops. Counts were made after 4 weeks of incubation.

RESULTS

Comparison of deep and surface counts

In the first experiment (Table 1) 10-day cultures of strains grown in 7H-3 + Tween + albumin medium were diluted 1/10⁵ in sterile distilled water and this dilution was inoculated on to plates and into bottles. The counts on

Table 1. *Surface and deep viable counts of Mycobacterium tuberculosis using 7H-3 medium*

χ^2 tests the conformance of the counts on the strains H37Rv, H37Ra, Branch and B.C.G. to a Poisson distribution.

Strain	Surface plate counts			Silica gel deep counts		
	No. of replicate drops	Mean count/drop	Viable units/ml.	No. of replicate bottles	Mean count/bottle	Viable units/ml.
H37Rv	6	13.3	6.67×10^7	5	141	7.05×10^7
H37Ra	9	6.3	3.17×10^7	4	65	3.25×10^7
Branch	6	31.3	1.57×10^8	4	343	1.71×10^8
B.C.G.	18	3.17	1.58×10^7	5	481	2.41×10^7
0139	10	29.9	1.50×10^8	5	32	1.60×10^7
B	10	2.4	1.20×10^7	5	1	5.00×10^5
$\chi^2 = 43.01$, D.F. = 35, $P = 0.5-0.3$				$\chi^2 = 11.98$, D.F. = 14, $P = 0.7-0.5$		

silica gel media of strains H37Rv, H37Ra, Branch and B.C.G. were slightly higher than the surface plate counts. The χ^2 indicates, in both methods, that the scatter of the individual counts followed a Poisson distribution. However, with strains 0139 and B, the silica gel counts were far lower than the surface plate counts. Growth of these two strains on both types of media was extremely poor, the colonies being minute even after 4 weeks of incubation. This finding suggested that they were particularly exacting in their growth requirements.

Cohn, Oda, Kovitz & Middlebrook (1954) showed that occasional strains of *Mycobacterium tuberculosis* are very exacting in their nutritional requirements and that isoniazid-resistant strains require a high iron content, pyruvic acid or catalase for their growth. The effect of adding silica sol on the iron concentration and the pH value of the medium was therefore examined. The addition of the sol, which had a pH value of 3.02, decreased the pH value of the medium

Table 2. *Surface and deep viable counts using 7H-3 and Kirschner medium*

Strain	Medium	Surface plate counts			Silica gel deep counts		
		No. of replicate drops	Mean count/drop	Viable units/ml.	No. of replicate bottles	Mean count/bottle	Viable units/ml.
H37 Ra	7H-3	9	7.34	3.67×10^7	5	90.2	4.51×10^7
	Kir.	5	3.41	1.70×10^7	5	39.2	1.96×10^7
H37 Rv	7H-3	17	3.53	1.76×10^7	6	42.2	2.11×10^7
	Kir.	7	1.43	7.15×10^6	6	43.7	2.18×10^7
B	7H-3	9	16.79	8.38×10^7	4	144	7.20×10^7
	Kir.	6	2.17	1.08×10^7	3	2.3	1.16×10^6
0704	7H-3	13	3.77	1.88×10^7	5	48.8	2.44×10^7
	Kir.	8	5.38	2.68×10^7	5	51.4	2.57×10^7
0942	7H-3	18	6.78	3.39×10^7	6	80.8	4.04×10^7
	Kir.	3	7.00	3.50×10^7	6	78.4	3.92×10^7
0435	7H-3	12	8.25	4.12×10^7	4	231	1.16×10^8
	Kir.	12	7.33	3.66×10^7	5	210	1.05×10^8
7H-3 medium		$\chi^2 = 70.22$, D.F. = 72, $P = 0.91$			$\chi^2 = 15.16$, D.F. = 25, $P = 0.95-0.90$		
Kirschner medium		$\chi^2 = 103.9$, D.F. = 35, $P = 0.001$			$\chi^2 = 34.49$, D.F. = 22, $P = 0.05-0.02$		

Analysis of variance

	Degrees of freedom	Mean square	Variance ratio	P
Between methods	1	11.4955	94.8	< 0.001
Between media	1	17.5275	144.6	< 0.001
Between strains	5	16.5896	136.9	< 0.001
Methods-media interaction	1	0.0260	0.2	> 0.2
Methods-strains interaction	5	8.4747	69.9	< 0.001
Media-strains interaction	5	8.0724	66.6	< 0.001
Error	5	0.1212	—	—

from 7.25 to 6.83 and removed by adsorption approximately half of the ferrous ions. In a second experiment the ferrous ammonium citrate concentration was therefore increased to 0.05 % and the pH value of the medium used for the plates adjusted to 6.8 by the addition of N-HCl. In this experiment Kirschner medium and 7H-3 medium were compared, both as plates solidified with agar and as silica gel deep cultures. The results together with an analysis of variance are shown in Table 2. The χ^2 on the counts in 7H-3 medium indicated conformity with a Poisson distribution, but those in Kirschner medium failed to conform. Counts by the silica gel method were significantly

higher than those on plates and they were also significantly higher in 7H-3 medium than in Kirschner medium. The highly significant interactions between methods and strains, and between media and strains indicates that the differences between the counts by the two methods and in the two media depended on the choice of strain. Strain B grew very poorly both on plates and in deep cultures of Kirschner medium. The analysis of variance was therefore repeated with the omission of the results using this strain, but the same terms were still significant.

Effect of diluent

The use of distilled water as a diluent was investigated by carrying out viable counts on a $1/10^5$ dilution of 10-day cultures grown in 7H-3 + Tween + albumin liquid medium, with distilled water or 0.1 % bovine albumin as diluents. The results of one of these experiments, with the H37Rv strain, is shown in Table 3. There was no decrease in the count over a 24 hr. period. A second experiment with strain B yielded similar results. One can therefore conclude that the results of the first two experiments were not influenced by the use of distilled water as a diluent, particularly as counts were always set up within 15 min. of dilution.

Table 3. *Viable counts on strain H37Rv after contact with distilled water or 0.1 % bovine albumin*

Time	Diluent	
	0.1 % bovine albumin	Distilled water
	\log_{10} number of viable units/ml.	
0	*	7.81
15 min.	7.85	7.85
30 min.	7.87	7.82
2 hr.	7.97	7.83
4 hr.	7.94	7.84
8 hr.	7.96	7.85
24 hr.	7.97	7.80

* Contaminated.

Further use of the silica gel method

We have used the silica gel method for the past two years in over 3000 counts of cultures and of homogenates of animal tissue. The method has been convenient, accurate and associated with a contamination rate of less than 1 %. Comparisons of 7H-3 medium and the oleic acid medium used by Fenner (1951) have shown that counts were the same, within experimental error, but colonies were slightly larger in the 7H-3 medium.

DISCUSSION

Surface viable counts of *Mycobacterium tuberculosis* on plates suffer from four disadvantages: (1) The contamination rate is high, particularly in certain laboratories where fungal spores are prevalent. (2) Closely spaced colonies tend to coalesce and are difficult to count. This necessitates the use of closely spaced

dilution intervals and numerous replicates. Alternatively, counts can be read with a plate microscope when the colonies are small, but the counts then tend to be low. (3) Plates must be poured the day before or the same day as the counts are carried out. (4) The opening of plates to read the counts may be a source of laboratory infections. Contamination is greatly reduced by seeding small bottles of medium with single drops, but the accuracy of this method is diminished by the coalescence of colonies which cannot be differentiated by the use of a plate microscope.

The deep counts of *Mycobacterium tuberculosis* in silica gel medium have the following advantages. The contamination rate is low, and there is no coalescing of colonies so that accurate counts can be made on bottles containing large numbers of colonies and wide dilution intervals are possible. The medium can be prepared well in advance of its use and the bottles need not be opened for counting. The dilution of drugs or other substances carried over in the inoculum is accurately known. The two disadvantages are that deep colonies have a less characteristic colonial morphology than surface colonies and it is more difficult to subculture from deep colonies. However, these disadvantages are of no great importance in the majority of experiments.

Deep counts in silica gel medium were higher than those on plates. Malachite green was used in the 7H-3 medium plates, but not in the silica gel bottles because it has been recommended as a procedure to decrease contamination. Although it remains possible that this dye may have been responsible for the lower counts, it seems unlikely as Cohn *et al.* (1954) stated that the concentration used was not inhibitory. Furthermore, phenol red was used in plates and bottles in Kirschner medium and the methods-medium interaction in the analysis of variance in the second experiment was not significant. This suggests that the incorporation of Malachite green in the 7H-3 medium did not contribute to the lower counts on the plates.

Miles & Misra (1938) found that the χ^2 distribution of surface counts was an indication of the quality of the medium. The results of the analysis of variance in Expt. 2 on the size of the counts and the values of χ^2 suggest that 7H-3 medium is superior to Kirschner medium.

Although the sterile distilled water used in these experiments had no bactericidal activity over a period of 24 hr., this cannot be accepted as a generalization to other laboratories for, as Wilson (1935) has shown, the bactericidal activity of distilled water from different stills varies.

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A Cell-wall Lytic Enzyme Associated with Spores of *Bacillus* Species

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SUMMARY: Aqueous extracts of disintegrated spores of *Bacillus cereus* and non-virulent *B. anthracis* contained an enzyme which produced visible lysis of the isolated cell walls of vegetative *B. cereus*. Optimum activity occurred at pH 7-8 in the presence of cobalt or manganese ions (10 p.p.m.) at 58°. Activity was destroyed during heating at 100° for 15 min. The lytic preparation released non-dialysable components containing α -diaminopimelic acid (DAP), glutamic acid, alanine, amino sugars and glucose. Although lysis was less obvious, the enzyme preparation released similar material from cell walls of other *Bacillus* species, spore coats of *B. megaterium* and coats of autoclaved *B. cereus* spores. Extracts of freshly harvested *B. cereus* spores were more active than those from spores which had been stored for several weeks at 2°. Extracts from disintegrated spores of *B. megaterium* had no enzymic activity; the enzyme system was associated with the insoluble spore coat fraction. The action of the enzyme differed from that of lysozyme or glucosaminidase; the reaction products did not give a significant reaction for *N*-acetylhexosamine and visible lysis proceeded more rapidly with cell walls of *B. cereus* than with *B. megaterium*. Possible functions of the enzyme may be to release 'spore peptide' from the spore coat during germination and to lyse the sporangium and free the spore during sporulation.

Spore germination in *Bacillus* species is accompanied by the excretion of calcium dipicolinate, amino acids and a non-dialysable peptide containing DAP, glutamic acid, alanine and amino sugars (Powell & Strange, 1953; Powell, 1953; Strange & Powell, 1954; Strange & Dark, 1956*a*). It has been suggested (Strange & Dark, 1956*b*) that the peptide is a constituent of the spore coat from which it is freed during germination or mechanical disruption, by the action of an enzyme present in the spore. Strange & Dark (1956*b*) found that spore-coat preparations of *Bacillus megaterium* still contained considerable amounts of peptide which was slowly and spontaneously released into water and buffer solutions. Similar preparations from *B. cereus* and *B. subtilis* contained only small amounts of peptide. Evidence for the build up of an intracellular enzyme system in sporulating *B. cereus* has recently been found by Powell & Strange (1956). The hexosamine and DAP content of extracts of *B. cereus* increased as sporulation proceeded; so also did their ability to split off from vegetative cell walls a complex containing hexosamine and DAP.

The present paper describes a partial purification of the enzyme system, some of its properties, and the products of its activity. A brief account of some of this work has already been presented (Strange & Dark, 1956*c*).

METHODS

Organisms. Vegetative organisms and spores of a laboratory strain of *Bacillus cereus* were grown in potato extract medium (Robinow, 1951) enriched with 1/10th vol. of casein hydrolysate + yeast extract (Gladstone & Fildes, 1940) as described by Powell & Strange (1956). Vegetative cells of laboratory strains of *B. megaterium* and *B. subtilis* were grown in tryptic meat broth. Thompson bottles containing 200 ml. of medium were inoculated with 1 ml. spore suspension (c. 10^7 spores) and shaken for 16–18 hr. at 37°. The vegetative organisms were washed twice with 0.9 % (w/v) saline and twice with distilled water by centrifugation at 0°.

Spores of *Bacillus cereus* were washed 5 times by centrifugation in saline and once in water. They were resuspended in water and stored for 4–7 days at 2°. After one more washing with water, stained films of the suspension were made. Vegetative debris was usually absent at this stage but if this persisted, the suspensions were stored for a further period at 2° and rewashed with water. Spores of an avirulent strain of *B. anthracis* (Sterne, 1937) were obtained by cultivation in shaken Thompson bottles containing 200 ml. of casein hydrolysate + yeast extract medium (Gladstone & Fildes, 1940) for 44 hr. at 37°. Clean spore suspensions of this organism were obtained as described for *B. cereus*.

Preparation of vegetative cell walls. Suspensions of freshly harvested vegetative organisms (10 mg. dry wt./ml.) were disintegrated in the Mickle (1948) tissue disintegrator with glass beads (Ballotini, size 12, from Chance Bros. Ltd.) and cell walls were obtained essentially as described by Salton & Horne (1951). The isolated cell walls were washed twice with saline and twice with distilled water and freeze-dried. Microscopic examinations were made of wet films of the preparations in dilute methylene blue. A preparation from *Bacillus cereus* was also examined under a Siemen's electron microscope with a magnification $\times 10,000$ by Mr W. F. Harris of the Chemical Defence Experimental Establishment. As far as could be judged by these methods, the preparations consisted essentially of cell walls.

*Preparation of crude enzyme solution from spores of Bacillus cereus
and B. anthracis*

Batches of spore suspension containing 10–25 mg. dry wt./ml. (16 hr., 100°) were disintegrated in the Mickle tissue disintegrator with Ballotini, size 12, for 45 min. at 2°. The suspension was centrifuged at 6,000 g. for 20 min. and the supernatant fluid filtered through a sterile sintered glass filter (porosity, 5/3). To obtain a solution of lytic enzyme free from spore peptide, thus avoiding a high 'blank value' in the lytic tests (see below), the filtrate was cooled to 2° and treated with an equal volume of cold, McIlvaine's sodium phosphate + citric acid buffer (pH 3.0). After standing at 2° for 15 min. the precipitate was centrifuged and washed with cold buffer (pH 3.0). The washed precipitate, which contained most of the lytic enzyme, was dissolved in McIlvaine's buffer (pH 7.4) and the volume adjusted with buffer to 1/4 that of the filtered spore

extract. This solution contained enzyme from 40 to 100 mg. (dry wt.) of spores and its hexosamine content determined after hydrolysis with 6 N-HCl was negligible. The buffered solution was freeze-dried and stored without loss of activity.

Determination of activity of crude enzyme preparations. Owing to the spontaneous lysis of cell-wall suspensions of vegetative *Bacillus cereus* (see Results) they were not satisfactory for the determination of the activity of the spore enzyme. This difficulty was avoided when cell-wall suspensions were heated at 100° before addition of enzyme solution. Freeze-dried cell walls of *B. cereus* were suspended in water (6 mg./ml.) and heated in a boiling water bath for 15 min. The suspension was centrifuged and the cell walls resuspended in water at the original concentration. A measured volume of cell-wall suspension (0.5 ml.) was treated with enzyme solution (0.25 ml.), water (0.25 ml.) and one drop of toluene. The tube was sealed and incubated in parallel with control tubes containing cell walls without enzyme and enzyme without cell walls. Temperature and time of incubation varied; details are given in Results. Measurement of enzymic activity by comparison of optical density readings was not generally adopted because (1) during incubation material precipitated out from the enzyme solution, (2) cell-wall suspension often contained floccules which sedimented rapidly, (3) dilute enzyme solutions did not produce a measurable effect. Instead, hexosamine was determined in the supernatant fluid after hydrolysis with 6 N-HCl for 2 hr. at 100° as described by Powell & Strange (1956). No hexosamine was found in the supernatant fluid from a suspension of heated *B. cereus* cell walls incubated without enzyme and the amount present in the enzyme solution itself was negligible. The hexosamine content of freeze-dried vegetative cell walls of *B. cereus* after hydrolysis with 6 N-HCl for 2 hr. at 100° was 30–35 % (as glucosamine) so that the maximum amount possible in the test supernatant was *c.* 1 mg./ml.

Analytical methods. Amino acids and amino sugars were detected by two-dimensional paper chromatography (Consden, Gordon & Martin, 1944) after hydrolysis with 6 N-HCl for 16 hr. at 100° in a sealed tube. The substituted amino sugar (Strange & Dark, 1956*a*; Strange, 1956) was separated from hexosamine by ascending paper chromatography with a mixture of *tert*-butanol/6 N-HCl/H₂O in the volume ratio 70:1:29 as solvent, and the amino sugars were detected with ammoniacal AgNO₃ (Partridge, 1948). Sugars were detected by single-dimensional paper chromatography after hydrolysis with N-HCl for 16 hr. at 100° in a sealed tube, aniline hydrogen phthalate being used as the spray reagent (Partridge, 1949). In all cases material for paper chromatography was freed from acid by evaporation *in vacuo* over sulphuric acid and caustic soda. Nitrogen was determined by a micro Kjeldahl-nesslerization method, phosphorus by the method of King (1932), total carbohydrate (excluding hexosamine) by the method of Sorensen & Haugaard (1933), hexosamine by the method of Elson & Morgan (1933) as modified by Immers & Vasseur (1950) after hydrolysis of the material with 6 N-HCl for 2 hr. in a sealed tube, and *N*-acetyl hexosamine by the method of Morgan & Elson (1934). DAP was estimated by means of the Chinard (1952) reaction, a method suggested by

Dr Elizabeth Work. A solution of hydrolysed material containing not more than 100 μg . DAP/ml. was heated with the acid ninhydrin solution for 5 min. at 100°. After dilution, the optical density was measured in a Unicam quartz spectrophotometer at 430 $m\mu$. and compared with standards treated similarly. None of the cell-wall components examined contained interfering amino acids. Paper electrophoresis examinations were made in the tank designed by Flynn & De Mayo (1951) on Whatman no. 4 paper using veronal/veronal sodium buffer (pH 8.6) and a voltage of 200. The components were detected with naphthalene black.

RESULTS

Spontaneous lysis of cell-wall suspensions

The tendency of suspensions of cell walls to lyse spontaneously increased as sporulation proceeded in the organisms from which they were obtained. Freeze-dried cell walls were prepared from *Bacillus cereus* grown in potato extract medium for various times. Duplicate amounts (3 mg.) were suspended in 0.02 M-phosphate buffer (pH 7.4; 1 ml.) with one drop of toluene and

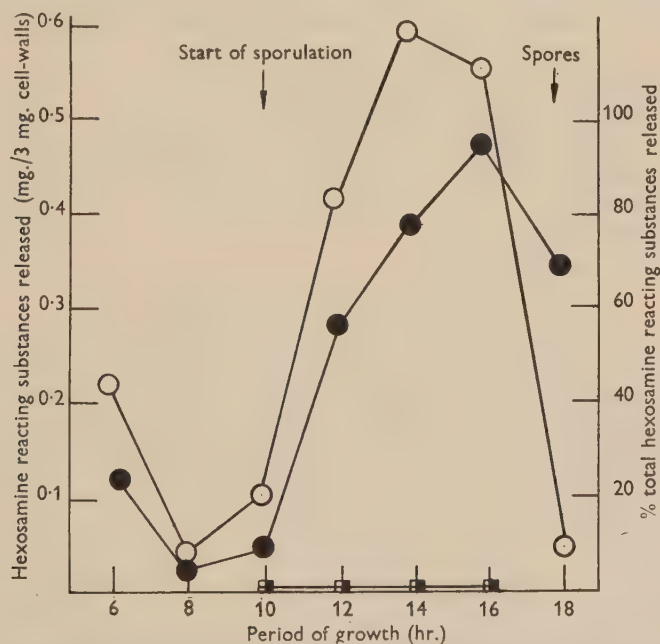


Fig. 1. Spontaneous lysis of *Bacillus cereus* cell walls suspended in 0.02 M-phosphate buffer (pH 7.4) for 16 hr. at 37°, in terms of the amount of hexosamine found in the acid-hydrolysed supernatant fluid; mg. hexosamine/3 mg. cell walls (O); % total hexosamine released (●). Release of hexosamine from cell walls heated at 100° for 15 min. (■).

incubated for 16 hr. at 37°. The extent of dissolution of each preparation, expressed in terms of the quantity of hexosamine found in the hydrolysed supernatant fluid, is shown in Fig. 1. In the medium used, the first signs of sporulation were evident after 10–12 hr. of incubation at 37° and the process was almost complete after 18 hr. Spontaneous dissolution of walls from

organisms harvested after 6–10 hr. growth was slight and only 100 μg . hexosamine was released. With walls of organisms harvested after 14 hr. growth, the degree of dissolution was considerable and 600 μg . hexosamine was found in the supernatant fluid. Thus in the tests of enzymic activity, substrate degradation could be decreased by selection of non-sporulating organisms; it was completely avoided by heating the preparations for 15 min. at 100° (Fig. 1). Such treatment did not affect the subsequent action of the spore enzyme.

*The effect of storage on the amount of extractable enzyme
in Bacillus cereus spores*

It was found that a suspension of viable *Bacillus cereus* spores which had been stored at 2° for one year provided a less active enzyme solution than was obtained from a freshly harvested spore suspension. A clean spore suspension (24 mg. dry wt./ml.) was prepared and stored at 2°. At intervals of 7 days, spore extracts were prepared and their enzymic activities determined (Table 1). After 7 weeks the extractable activity had fallen to 20 % of that present in extracts from freshly harvested spores. For most of the experiments described in this paper, enzyme solutions were prepared from freshly harvested spores.

Table 1. *Decrease in extractable enzyme in Bacillus cereus spores
after storage*

Buffer: 1:4 McIlvaine's (pH 7.4); substrate: 3 mg. *Bacillus cereus* cell walls; lytic enzyme from 24.0 mg. *B. cereus* spores; incubation 44 hr. at 37°; toluene as preservative; duplicate tests.

Age of suspension (weeks)	...	1	2	3	5	6	7	8	52
Substrate hexosamine released (%)		91	78	52	35	23	19	22	12

Optimum pH for enzymic activity

A series of precipitates obtained at pH 3.0 from 10 ml. volumes of extracts of *Bacillus cereus* spores (22.6 mg. dry wt./ml.) were suspended or dissolved in 2.5 ml. volumes of McIlvaine's buffer of different pH values and the activity of the buffered enzyme preparations determined (Table 2). The optimum pH value for enzymic activity was between 7.0–8.0 and as the pH value of whole spore extract was 7.4, this value was used in lytic tests.

Table 2. *Effect of pH value on the activity of Bacillus cereus spore enzyme*

Buffers: 1:4 McIlvaine's; substrate: 3 mg. *Bacillus cereus* cell walls; lytic enzyme from 23 mg. *B. cereus* spores; incubation: 44 hr. at 37°; toluene as preservative; duplicate tests.

pH value	...	4.25	5.3	6.2	7.1	7.7
Substrate hexosamine released (%)		36	26	59	89	88

Stimulation of enzymic effect by metal ions

The effect of each of the following metals on the lytic activity of *Bacillus cereus* spore enzyme was tested by adding them as sulphates to *B. cereus* cell walls (3 mg.) and enzyme from 12 mg. *B. cereus* spores (final concentration of metal ion, 10 μg ./ml.): Mn^{++} , Fe^{++} , Fe^{+++} , Mg^{++} , Co^{++} . The suspensions were

incubated at 37° for 18 hr. in parallel with suspensions without additions. The results given in Table 3 show that cobalt had a marked and manganese a moderate enhancing effect on the release of substrate hexosamine, but that ferrous or ferric iron and magnesium had no effect. The McIlvaine's buffer used in these experiments contained citric acid which is known to form non-dissociated complexes with metals. To avoid such an effect, the experiment was repeated using 0.025 M tris-(hydroxymethyl)-aminomethane HCl buffer (Gomori, 1946) and extended to investigate the effect of copper and nickel ions. The results (Table 4) showed that cobalt and manganese and to a lesser extent copper and nickel ions had a stimulating effect on hexosamine release.

Table 3. *Effect of metal ions on the lytic effect of Bacillus cereus spore enzyme*

Buffer: 1:4 McIlvaine's (pH 7.4); substrate: 3 mg. *Bacillus cereus* cell walls; lytic enzyme from 12 mg. *B. cereus* spores; metal ions: 10 µg./ml.; incubation: 18 hr. at 37°; toluene added; duplicate tests.

Metal ion addition	...	Nil	Mn ⁺⁺	Fe ⁺⁺	Fe ⁺⁺⁺	Mg ⁺⁺	Co ⁺⁺
Substrate hexosamine released (%)		63	70	61	66	64	92

Table 4. *Effect of metal ions on the lytic effect of Bacillus cereus spore enzyme in the presence of an organic buffer*

Buffer: 0.025 M-tris-(hydroxymethyl)-aminomethane HCl; other details as in Table 3.

Metal ion addition	...	Nil	Mn ⁺⁺	Fe ⁺⁺	Fe ⁺⁺⁺	Mg ⁺⁺	Co ⁺⁺	Cu ⁺⁺	Ni ⁺⁺
Substrate hexosamine released (%)		28	51	21	20	26	53	38	36

Inhibition and destruction of enzymic effect

The effect of each of the following substances was tested in duplicate by adding them to cell walls (3 mg.) and enzyme from 12 mg. *Bacillus cereus* spores; KCN (mM, 10 mM), chloroform (2 drops), NaF (25 mM), thiomersalate (1:5000), 8-hydroxyquinoline (oxine; mM). The values given in brackets are final concentrations in a volume of 1 ml. The effect of heating the enzyme solution at 60° for 1 hr. and at 100° for 15 min. was also investigated. The series was incubated at 37° for 16 hr. with tests without additions and controls. Results of determinations of enzymic activity are given in Table 5, which shows that: (1) activity was destroyed by heat at 100° for 15 min. but not at 60° for 1 hr.; (2) activity was inhibited by the presence of 10 mM-KCN but was unaffected by mM-KCN; (3) some inhibition occurred in the presence of mM-oxine.

Optimum conditions for enzymic activity

Rapidly visible lysis of walls of vegetative *Bacillus cereus* occurred when the walls were incubated at 58° with buffered enzyme solution (pH 7.4) containing cobalt (10 µg./ml.). Enzyme from 12 mg. *B. cereus* spores released 75–85 % of the hexosamine present in 3 mg. of cell walls in 2 hr. No hexosamine was released from heated cell walls incubated under these conditions in

the absence of enzyme solution. The enzyme from 26, 13, 6.5 and 3.25 mg. dry weight of spores released 77, 70, 35 and 18 %, respectively, of the substrate hexosamine.

The effect of lytic enzyme from various sources on different substrates

The activity of an enzyme solution from *Bacillus cereus* spores was determined with vegetative cell walls of *B. cereus*, *B. subtilis* and *B. megaterium* as substrates (Table 6). Spontaneous degradation of suspensions of *B. subtilis* walls was decreased but not stopped by heating them at 100° for 15 min. and this effect was taken into account in the results. Hexosamine-containing material was released by the enzyme in all cases.

Bacillus cereus spore enzyme solution also had a degradative effect on preparations of coats of *B. megaterium* and of autoclaved *B. cereus* spores (Table 6). The preparation of *B. megaterium* spore coats used had been stored in a dried state for several months, and in contrast to the behaviour of freshly prepared spore coats, no spontaneous release of hexosamine-containing material occurred. Coats of viable *B. cereus* spores contained 2-3 % hexosamine, and the release of hexosamine-containing material either spontaneously or in the presence of lysozyme was small (Strange & Dark, 1956*b*). It appeared that on disintegration of spores of *B. cereus*, the enzyme removed 'spore peptide' almost completely from the spore coat. It was found, however, that coats isolated from *B. cereus* spores autoclaved at 15 lb./sq.in. for 20 min. contained 8.3 % hexosamine. A significant amount of hexosamine-containing material was released from them by the enzyme solution.

Extracts from *Bacillus anthracis* spores had a similar activity to those from *B. cereus* spores. No activity was detected in extracts prepared from *B. megaterium* spores. With *B. megaterium* it seemed likely that a similar enzyme must be present in the spore since it was previously shown that hexosamine-containing material was released from freshly isolated spore coats suspended in buffer solution (Strange & Dark, 1956*b*). The enzymic activity of spore coat preparations of *B. megaterium* was therefore tested. Spore coats from 40 mg. of dried fresh spores were suspended in 1 ml. McIlvaine's buffer (pH 7.4) and dispersed by shaking in the Mickle tissue disintegrator for 1 min. The suspension (0.25 ml.) was added to 3 mg. *B. cereus* vegetative cell walls suspended in 0.75 ml. water and after the addition of toluene, the mixture was incubated at 37° for 16 hr. on a rotary shaker. After correcting for the hexosamine-containing material released from the spore coats themselves, it was concluded that a small but definite release of material had occurred from *B. cereus* cell walls (Table 6).

Partial purification of the crude lytic enzyme

The crude enzyme preparation contained most of the spore nucleoprotein. Attempts to purify the enzyme by precipitation with ammonium sulphate were not successful since most of the material was precipitated at a low salt concentration. However, addition of clupein sulphate caused precipitation of a nucleoprotein complex and the filtrate, containing most of the enzymic

Table 5. *Destruction and inhibition of enzymic effect of Bacillus cereus spore enzyme*

Buffer: McIlvaine's (1:4; pH 7.4); substrate: 3 mg. *Bacillus cereus* cell walls; lytic enzyme from 12 mg. *B. cereus* spores; incubation: 16 hr. at 37°.

Addition or treatment	Chloroform	NaF	Thiomersalate 'Oxine'	60°/1 hr. 100°/15 min.	Nil
Concentration	2 drops	25 mm	1:5000
Substrate hexosamine released (%)	55	54	50	50	53
			14				8
			51				

Table 6. *Effect of enzyme from various sources on different substrates*

Buffer: 1:4 McIlvaine's (pH 7.4); total volume in tests = 1 ml.; incubation at 37° or 60°.

Source of enzyme	Substrate	Substrate hexosamine (%)	Co++ (10 p.p.m.)	Incubation time (hr.)	Substrate hexosamine released (%)
10 mg. <i>Bacillus cereus</i> spores (ext.)	3 mg. <i>B. cereus</i> cell walls (H)*	30	+	2 (60°)	78
24 mg. <i>B. cereus</i> spores (ext.)	3 mg. <i>B. subtilis</i> cell walls (H)	21	-	16 (37°)	31
32 mg. <i>B. cereus</i> spores (ext.)	3 mg. <i>B. megaterium</i> cell walls (H)	12	+	16 (37°)	55
32 mg. <i>B. cereus</i> spores (ext.)	10 mg. <i>B. megaterium</i> spore coats (old)	4.4	+	16 (37°)	31
24 mg. <i>B. cereus</i> spores (ext.)	3 mg. <i>B. cereus</i> spore coats (autoclaved)	8.3	+	2 (60°)	74
18 mg. <i>B. anthracis</i> spores (ext.)	4.5 mg. <i>B. cereus</i> cell walls (H)	30	-	44 (37°)	41
20 mg. <i>B. megaterium</i> spores (ext.)	3.0 mg. <i>B. cereus</i> cell walls (H)	30	+	16 (37°)	0
Spore coats from 10 mg. <i>B. megaterium</i> spores	3.0 mg. <i>B. cereus</i> cell walls (H)	30	+	16 (37°)	11

* (H) = heated at 100° for 15 min. ext. = extract.

activity, could then be fractionated with ammonium sulphate. A solution (8.5 ml.; pH 7.4) containing enzyme from 910 mg. dried spores was treated with 1.8 ml. of an aqueous solution of clupein sulphate (1 %, w/v). The viscous nucleoprotein complex was centrifuged and rejected. The supernatant fluid (9.3 ml.) was treated with saturated ammonium sulphate solution (3 ml.) and allowed to stand at 2° for 2 hr. The precipitate (I) was collected by centrifugation. A second precipitate (II) formed when the supernatant fluid was treated with more ammonium sulphate solution (6.3 ml. \equiv 0.5 saturation) and left at 2° for 16 hr. A third precipitate (III) formed on saturating the supernatant fluid from II with the solid salt. Each precipitate was dissolved in 9.3 ml. of McIlvaine's buffer (pH 7.4) and tested for enzymic activity. The release of hexosamine-containing material from 3 mg. *Bacillus cereus* cell walls by 0.25 ml. of solutions of I, II and III after incubation for 2 hr. at 60° in the presence of cobalt (10 p.p.m.) was 20, 86 and 38 % respectively, of the total. After dialysis and freeze-drying solution II was found to contain 0.75 mg. dry wt./ml. of material, indicating that 190 μ g. was present in the enzymic test. The freeze-dried, dialysed preparation, on reconstitution in buffer, was not as active as the original solution, indicating that inactivation had occurred. No loss of activity occurred when buffered solutions of II were freeze-dried.

The composition of the material released from vegetative cell walls

Material for analysis was obtained by the action of enzyme from *Bacillus cereus* spores on vegetative cell walls of *B. cereus*, *B. megaterium* and *B. subtilis*. Details of the isolation procedure used are given in the following typical experiment. *B. cereus* cell walls (80 mg.) were suspended in water (6 ml.) and heated at 100° for 15 min. The suspension was centrifuged and the cell walls resuspended in McIlvaine's buffer (pH 7.4) diluted 1:4 (8 ml.) containing enzyme from 200 mg. spores + 100 μ g. cobalt. After incubation for 2 hr. at 58° followed by 16 hr. at 37° the undigested material was removed by centrifugation and the supernatant fluid cooled to 0°. Trichloroacetic acid solution (25 %, w/v) was added to a concentration of 3 % (w/v) and the mixture allowed to stand 30 min. at 0°. After removal of the precipitate by centrifugation, the supernatant fluid was neutralized with solid NaHCO_3 and shaken with chloroform + amyl alcohol (Sevag, 1934) to remove remaining traces of protein. The protein-free solution was dialysed in the cold and freeze-dried. The yield of white solid was 40–45 mg. From 100 mg. of cell walls of *B. megaterium* or *B. subtilis*, 14–20 mg. of product was obtained. Material released from cell walls by the enzyme is subsequently referred to as peptide.

After acid hydrolysis followed by paper chromatography, the following constituents were found to predominate in all cases; DAP, glutamic acid, alanine, hexosamine, another amino sugar and glucose. The other amino sugar has been found in a number of bacterial walls (Cummins & Harris, 1954, 1956) including those from *Bacillus* species (Salton, 1956) and appears to be identical with the one found in 'spore peptide' (Strange & Powell, 1954; Strange & Dark, 1956a). A possible structure, 3-O- α -carboxyethylhexosamine has been

suggested for it (Strange, 1956). Traces of other amino acids, principally glycine, serine and aspartic acid, were sometimes found. The analyses of the peptides given in Table 7 show that a large amount of hexosamine was present, varying from 37 to 47 %. Examination of *B. megaterium* cell-wall peptide by paper electrophoresis showed the presence of only one component staining with Naphthalene Black. The component moved rapidly towards the anode at

Table 7. *Analysis of material released by lytic enzyme from cell walls of Bacillus cereus, B. megaterium and B. subtilis*

	Total nitrogen	Hexosamine (after hydrolysis)	Total phosphorus	Total carbohydrate as glucose
	g./100 g. cell-wall			
<i>Bacillus cereus</i>	6-7	44-47	0.3-0.4	10-15
<i>B. megaterium</i>	5	37	0.6	7
<i>B. subtilis</i>	6.1	37	1.6	10

pH 8.6 and on elution from stained strips it was found to be similar in composition to the original peptide. On paper electrophoresis, *B. cereus* cell-wall peptide was found to contain several components moving towards the anode. Fractionation of an aqueous solution of the mixture with ethanol in the presence of barium acetate (1 %, w/v) gave a number of fractions, all of which contained the same constituents. The purest fraction obtained in 20 % yield after repeated alcohol fractionations contained 6.5 % nitrogen, 50 % hexosamine (as glucosamine), 10 % glucose, 0.27 % phosphorus and 3.2 % DAP. Paper electrophoresis of this fraction showed the presence of one major component moving towards the anode with a trace of a second, faster moving material. It appeared that the peptide material released by the lytic enzyme from *B. cereus* cell walls was a mixture of components with similar chemical composition but varying in the degree of polymerization. Glucose appeared to be part of the moiety and not present as a separate polysaccharide. No fraction free from phosphorus was obtained and the ultraviolet absorption spectrum of a solution of the fractionated material which contained 0.27 % phosphorus, indicated that only a small amount of this constituent was present as nucleic acid.

Peptide from *Bacillus megaterium* cell walls gave no significant reaction for *N*-acetylhexosamine, but after incubation with lysozyme a positive reaction was obtained. Greater degradation occurred in the presence of an extract of the gut of *Helix aspersa* and the optimum pH value for activity was 5.0: In a typical experiment 10 mg. of material was treated with 0.3 ml. of a clarified extract (10 ml. aqueous extract from gut of twenty snails), 0.2 ml. McIlvaine's buffer (pH 5.0) and 0.7 ml. water. After incubation for 16 hr. at 37° in parallel with controls (a) without peptide, (b) without snail enzymes, *N*-acetylhexosamine determinations were made on samples from the diluted centrifuged mixtures. Maximum colour, equivalent to 21 % *N*-acetylglucosamine, was obtained with Ehrlich's reagent after heating the test solution for 4 min. with

dilute alkali solution. *N*-acetylglucosamine also gave the maximum colour under these conditions, which suggested that the free *N*-acetylated amino sugar had been released (Aminoff, Morgan & Watkins, 1952). The reaction mixture was examined by paper chromatography and a spot was obtained which reacted with Ehrlich's reagent and had the same R_f value as *N*-acetylglucosamine. No reaction for *N*-acetylhexosamine was obtained with either control solution.

Under the above conditions very little *N*-acetylhexosamine-reacting material was released from *Bacillus cereus* cell-wall peptide by snail enzymes; lysozyme also had no effect. *N*-Acetylated amino sugars were present in this peptide since after hydrolysis with *N*-acetic acid the products gave a positive Morgan & Elson reaction and it appeared that the amino sugar linkages present differed from those in *B. megaterium* cell-wall peptide.

During these experiments it was found that snail enzyme lysed viable vegetative *Bacillus subtilis* at least as rapidly as lysozyme but had no effect on its spores.

DISCUSSION

Greenberg & Halvorson (1955) reported the presence of an enzyme in culture filtrates of *Bacillus cereus* var. *terminalis* which lysed whole vegetative cells of this organism and of another strain of *B. cereus*. The enzyme was relatively heat stable and developed during the sporulation stage. Its activity was stimulated by the presence of manganese. These workers were unable to demonstrate enzymic activity in extracts of disintegrated cells and no evidence is given that the cell-wall was attacked. Nomura & Hosoda (1956) prepared an enzyme from lysates of vegetative *B. subtilis* which lysed the isolated cell walls of this organism. In the present work the enzyme found in extracts from spore suspensions freed from vegetative forms attacked vegetative cell walls and spore coats. The criterion used for activity was the release of soluble non-dialysable material which contained α -diaminopimelic acid, alanine, glutamic acid and amino sugars. These compounds predominate in cell walls from a variety of Gram-positive bacteria (Cummins & Harris, 1954, 1956) including *Bacillus* species (Salton, 1953*a*). In the presence of sufficient enzyme, digestion of *B. cereus* vegetative cell walls was almost complete, whereas with *B. megaterium* cell walls a considerable amount of undigested material remained. In contrast to this Salton (1953*b*, 1956) showed that lysozyme had considerably less effect on the cell walls of *B. cereus* than on those of *B. megaterium*.

The material released by the enzyme of the present work had a characteristic composition similar to that of 'spore peptide'. It appears that this type of complex forms part of the integuments of both vegetative forms and spores of Gram-positive organisms. Compared with spore peptide from the same organism, the analogous cell-wall component contains more glucose, and it is probable that this is part of the amino acid + amino sugar moiety. The amount of phosphorus found in *Bacillus megaterium* 'spore peptide' was negligible (0.03 %), but significant amounts were found in all cell-wall peptides and

purified fractions from them. In the present investigation it has not been possible to decide whether this is part of the peptide or a contaminant. Enzymes from snail gut are known to hydrolyse chitin and it is interesting to find that they split some of the cell-wall peptides. One of the enzymes present in snail gut preparations is β -glucosaminidase (Neuberger & Pitt Rivers, 1939) and perhaps β -glucosaminide linkages are present in peptide from *B. megaterium* cell walls but absent from the peptide of *B. cereus* cell walls or present to a much smaller extent. It has been found that *B. megaterium* 'spore peptide' is broken down to a much greater extent than that of *B. cereus* by snail enzymes.

The presence of the lytic enzyme in spores suggests a mechanism by which 'spore peptide' may be released from the spore coat on germination, especially as we have shown that the enzyme attacks coats of both *Bacillus megaterium* and autoclaved *B. cereus* spores. It is possible that the relatively large amount of 'spore peptide' found in spore coats of *B. megaterium* is related to the small amount of lytic enzyme present in contrast to the small amount of peptide in the spore coats and larger amount of enzyme in spores of *B. cereus*. Suspensions of cell walls prepared from sporulating cultures of *B. cereus* lysed spontaneously on incubation, probably due to the presence in them of the same or a similar enzyme, the activity of which was destroyed on heating at 100°. The function of this type of system in the sporulating cell may be to lyse the sporangium, thus releasing the spore.

Several explanations can be offered to account for the decreasing amount of extractable enzyme present in *Bacillus cereus* spores during storage. It is possible that the enzyme is not in fact intracellular, but strongly adsorbed on the outer surface, where it is slowly denatured. It was, however, found that freshly harvested spores which had been washed sixteen times with reagents including sodium bicarbonate and acetic acid still gave active extracts. Spores after storage for one year at 2° contained some extractable enzyme. It is possible that the freshly harvested spore contains an excess of the lytic enzyme and that only a relatively small proportion of this is incorporated or adsorbed on to the spore protoplasm, the remainder being slowly denatured and lost.

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An Apparatus for the Continuous Culture of Bacteria at Constant Population Density

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SUMMARY: A self-regulating continuous culture apparatus is described, designed for studying growth and enzyme production in bacteria which require complex media; it might also be suitable for the cultivation of suspended tissue cells. The growth vessel is a tilted cardioid-shaped flask spinning about its long axis. Aeration and mixing are achieved in such a manner that no frothing occurs and no 'anti-foam' agents are required. Adherent bacterial colonies do not form on the walls of the growth vessel. Samples are removed rapidly under aerobic conditions. The basal medium can be automatically augmented with special metabolites at constant concentration without contaminating the main medium reservoir. The apparatus is autoclaved in one unit after assembly. Factors governing the design of similar small-scale apparatus are discussed.

The fundamental theory of homocontinuous or capacity flow culture has already been established by numerous authors (Monod, 1950; Novick & Szilard, 1950*a, b*; Spicer, 1955; Herbert, Elsworth & Telling, 1956), and the principles governing the construction of a self-regulating apparatus for maintaining growing bacterial cultures at constant population density are generally agreed. The three basic requirements for aerobic organisms are: a growth vessel in which the culture is thoroughly mixed and aerated; a reservoir to supply fresh medium to the growth vessel at constant rate; a device to keep the volume of culture in the growth vessel constant, by removing culture at the same rate as the addition of fresh medium. However, since various ancillary devices are necessary if the apparatus is to operate accurately without too much attention, and since most operations must be performed at constant temperature under aseptic conditions, the final assembly is rather more complicated than its simple formulation suggests.

Each of the practical difficulties which arise can be resolved in various ways, and numerous assemblies for continuous culture have already been described (see the review by Novick, 1955; also De Haan & Winkler, 1955; Herbert *et al.* 1956), but none of them is the perfect all-purpose equipment. When designing a continuous culture method it is therefore necessary to take into account the particular purpose for which it is to be used. An excellent apparatus for mass-production of bacteria may be quite unsuitable for studies of mutation rates or cell metabolism. The pathogenicity of the organism, its nutritional requirements and growth rate also affect the design of the apparatus.

The apparatus described here was designed primarily for studying the induced formation of penicillinase in broth cultures of *Bacillus cereus* at growth

densities equivalent to 1–2 mg. dry wt. organisms/ml. culture. The proposed experiments demanded the following conditions: (1) easy sterilization and the ability to operate with little chance of contamination; (2) low medium consumption; (3) aeration without foaming; (4) absence of adherent bacterial colonies from the walls of the growth vessel; (5) contact of medium and culture only with chemically inert materials, such as glass and silicone; (6) rapid transfer of culture from growth vessel to sampling tube under aerobic conditions; (7) augmentation of the basal medium with special metabolites during operation, without contamination of the main medium reservoir.

A prototype apparatus was completed to these specifications in 1951 and has since worked satisfactorily. Details of its performance and the experimental results will be given in another paper. However, experience suggested improvements which have been incorporated in later models, and which are mentioned here after the description of the prototype. Finally, some of the factors governing the design of the components are discussed for the guidance of workers who may wish to adapt the equipment for their own purpose. It appears that similar apparatus might be particularly suitable for the continuous culture of suspended tissue cells.

GENERAL DESCRIPTION

The prototype apparatus is intended for operation in a hot-room, and is mounted on a non-rusting metal framework in such a manner that all parts requiring sterilization can be autoclaved in one unit after assembly. All glass components are made of 'Pyrex' glass with standard joints lubricated with silicone grease.

Fig. 1 shows in schematic form the functions of the various components and their interconnexions; Fig. 2 is a conventionalized diagram of the apparatus lettered to correspond with the detailed description given below; Fig. 3 shows details of the growth vessel; and Fig. 4 the associated electrical circuits.

Air supply

Medium enters the growth vessel under gravity and is removed by suction; since the growth vessel has a liquid seal, the air inside it must be at nearly atmospheric pressure. The apparatus therefore has two air-circuits; one operates above atmospheric pressure and supplies sterile humidified air, and the other operates below atmospheric pressure and withdraws air. These are referred to respectively as the positive and negative air lines: they are constructed of wide-bore tubing to eliminate flow pressure gradients, except at points where a constriction is inserted for some specific purpose.

Positive air line. Compressed air passes through a reservoir *Q* to the main filter *P* which is a 5 cm. diam. tube packed firmly with non-absorbent cotton wool. From *P* the air by-passes a combined blow-off valve and water manometer *O*, consisting of a wash-bottle with the inlet tube dipping 20 cm. into

water, and flows to the saturator *M*. The saturator is in effect a wash-bottle, with a 60 cm. neck serving as a reflux condenser. The incoming air is bubbled through water in a flask maintained at about 70° by a thermostatically-

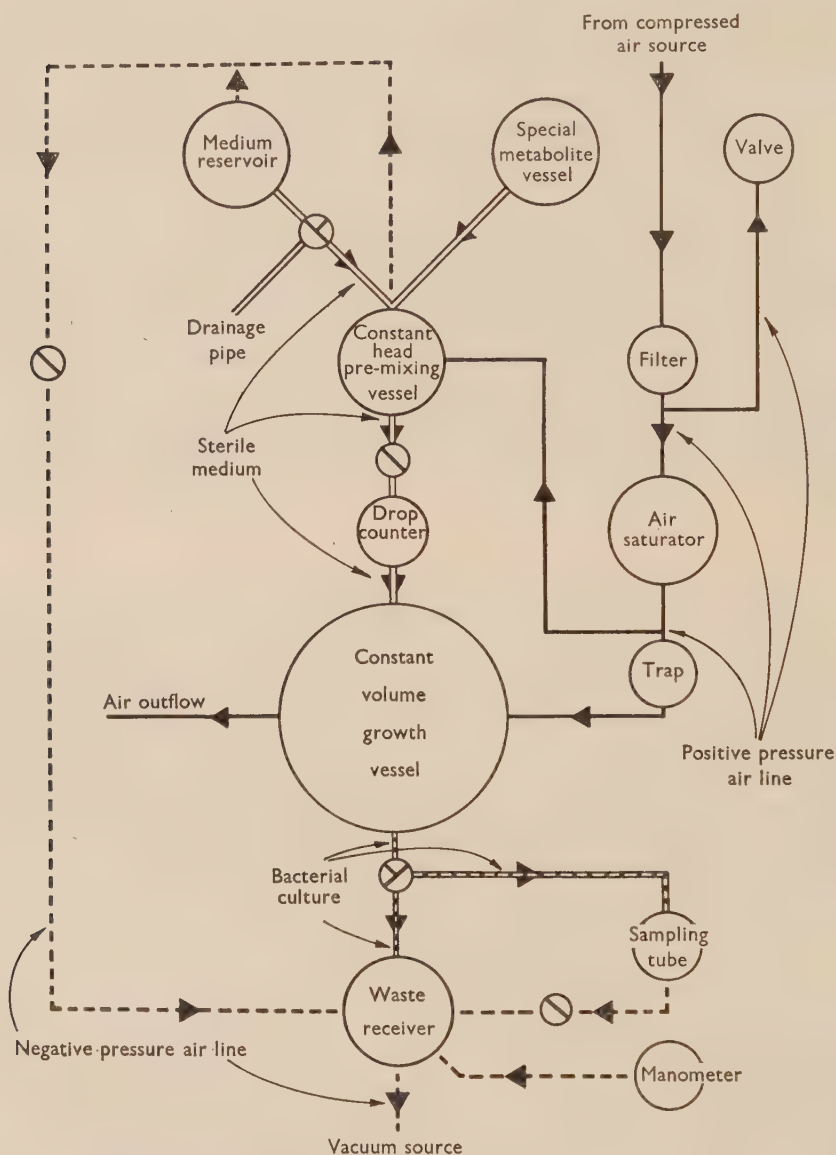


Fig. 1. Schematic diagram of the apparatus, showing the functions of the components and their interconnexions.

controlled heating mantle, and then cooled to ambient temperature as it rises up the neck. When necessary the flask can be topped-up with sterile water through a side-arm *N*.

A small proportion of the humidified sterile air emerging from *M* is diverted to operate the constant-head device in *B*; the majority passes to the trap *L* which is again a wash-bottle with the inlet tube immersed in water to a depth

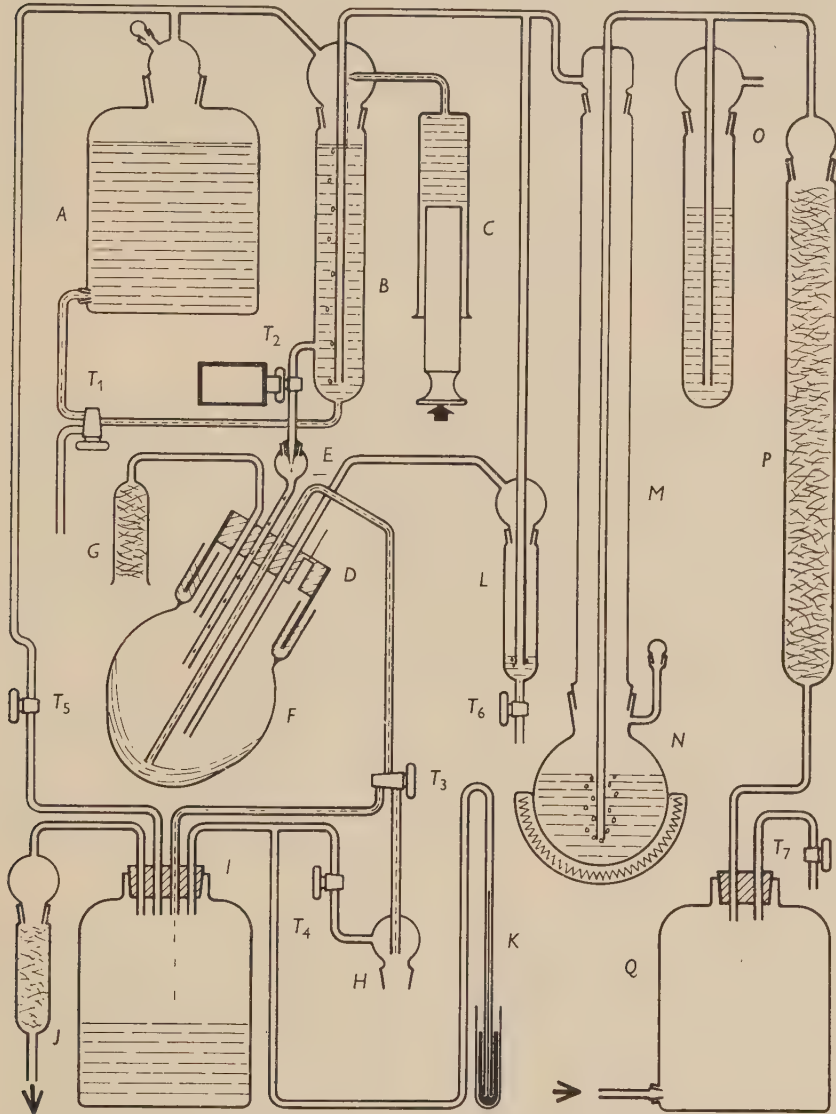


Fig. 2. Conventionalized diagram of the apparatus lettered to correspond with the description in the text. The components are not drawn to scale.

of 1 cm. only. If extra water collects in the trap it can be run off through tap *T*₆.

From the trap, air passes through the fixed sealing-head *D* to the growth vessel *F*, where it is blown over the surface of the culture. Most of the air is

then sucked out with excess culture through the constant-volume control tube, but some passes directly into the atmosphere through a large area cotton-wool, filter *G*.

Negative air line. A slight suction is maintained through a cotton-wool filter *J*, which removes aerosols, to a 10 l. aspirator *I* serving both as a low-pressure reservoir and as a receptacle for unwanted effluent culture from the growth vessel. Lysol is placed in the bottom of *I* to prevent further bacterial growth.

From *I* negative air lines run directly to a mercury manometer *K*, through a tap *T*₄ to the sampling head *H* and through a three-way tap *T*₃ to the constant-volume control tube in the growth vessel. A fourth connexion is made through a variable leak *T*₅, which is a grooved tap of the type shown in Fig. 5, to the medium reservoir *A* and constant-head vessel *B*.

Medium supply

The medium reservoir *A* is a 5 l. graduated aspirator, closed at the top by a head connected to the negative air line and carrying a stoppered refilling port through which the reservoir may be replenished while the apparatus is working. Medium flows from *A* under gravity through a tube fitted with a three-way drainage tap *T*₁ at its lowest point, entering at the bottom of the constant-head vessel *B* in which it rises to the same level as the liquid in *A*.

The constant-head vessel is a graduated tube of about 300 ml. capacity, slightly longer than the height of *A*. It is fitted with a wash-bottle head which is so connected to the positive and negative air lines that a slow stream of sterile air bubbles is drawn through the medium. The pressure at the tip of the bubbling tube is fixed above that in the growth vessel by the depth of water in trap *L*; i.e. about +1 cm. of water. The assembly operates as a modified Mariotte bottle supplying medium at a constant head to *T*₂ and is able to compensate rapidly for ambient temperature or pressure changes. In addition, the bubbles stir the contents of the vessel and mix any special metabolites being injected from *C*.

The special metabolite vessel *C* is a 100 ml. 'Pyrex' glass syringe driven by a pulsed stepwise motor through a screw thread. One pulse to the motor turns the screw through $\frac{1}{120}$ revolution and injects 0.003 ml. of metabolite solution through a fine capillary into *B*. Pulses are generated electrically at intervals continuously variable from 0.5 to 50 sec., and also at a fixed short interval of 0.05 sec.

Medium leaves *B* through a side-arm, in order to avoid any precipitate which may have collected at the bottom of the vessel, and flows under gravity to the adjustable flow resistance *T*₂. *T*₂ is a glass tap made leaky by cutting a pair of shallow circumferential grooves in its surface with a sharp triangular needle file (Fig. 5). From *T*₂ medium passes to *E* where it emerges in discrete drops from a calibrated jet and then, after running down the side of an inclined wide-bore tube through the fixed sealing head *D*, drips into the growth vessel.

The growth vessel and fixed head

The growth vessel (Fig. 3, and *F* in Fig. 2) is a cardioid-shaped flask of about 500 ml. capacity, having a broad neck with two concentric walls enclosing a 2:1 (v/v) glycerol + water mixture which seals it to the fixed head. It is mounted on a turntable, inclined at 30° from the vertical, so that it can be rotated about its long axis at constant speed. The drive is provided by a rheostat-variable 24 V., d.c. motor geared down to 400 rev./min. maximum speed. The fixed sealing-head is a glass or stainless-steel cylinder, open at the

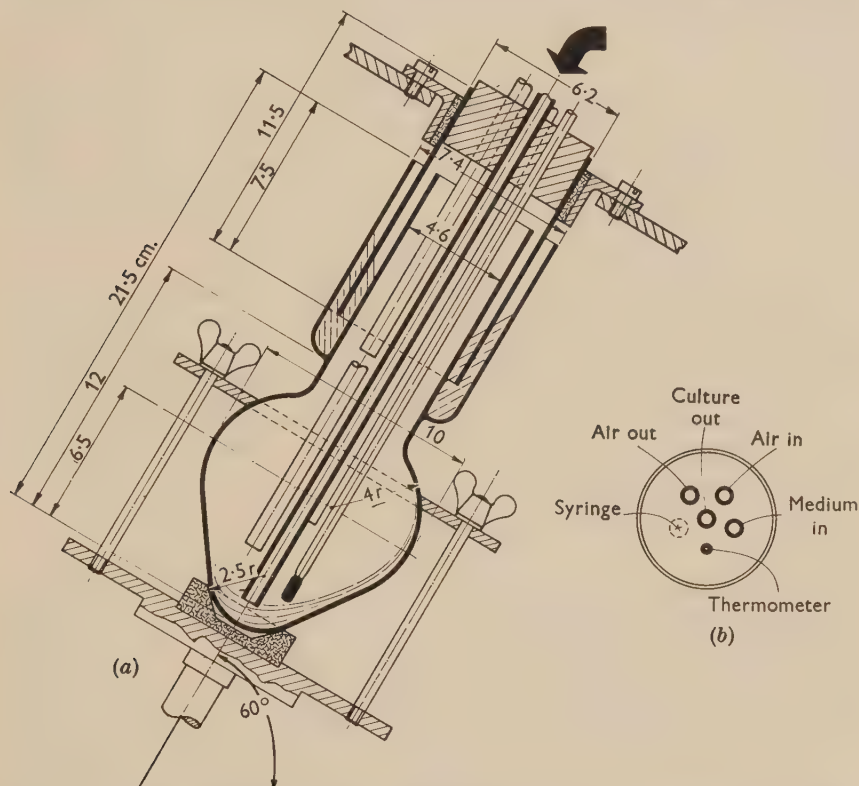


Fig. 3. (a) The growth vessel and parts of the associated equipment. The sealing-head shown is of the earliest type, being a glass cylinder closed at the top with a synthetic-rubber bung. (b) Plan of the top of the sealing-head, showing the disposition of the various apertures.

base and closed at the top except for a set of tubular sleeves, through which pass the various service tubes gripped by short lengths of rubber or silicone tubing. There is also an inoculation port stoppered with a soft-rubber cap to permit the passage of the needle of a hypodermic syringe. The fixed head is mounted on an adjustable gantry so that its long axis can be made to coincide with that of the spinning growth vessel.

During operation the growth vessel contains about 100 ml. of culture spun into a layer on the walls and base of the flask. Very good mixing results from the continual rapid transport of culture, once in each revolution, from top to

bottom of the flask. Drops of acid or alkali added to a solution of pH indicator in the flask appear to produce instantaneous colour changes throughout the liquid mass. There is no foaming even with the richest media, and the large surface area exposed provides aeration good enough to support cultures of *Escherichia coli* or *Bacillus cereus* equivalent to 1 mg. dry wt./ml. at a generation time of *c.* 25 min.

Culture disposal

The volume of culture is maintained constant in the growth vessel, so long as dilution rate and speed of rotation are constant, by a capillary tube of about 1.5 mm. bore passing through the centre of the fixed head to within about 1.5 cm. of the bottom of the vessel and connected indirectly to the negative air line. The rapid low-amplitude wobble of the culture surface causes the tip of the capillary to be immersed for a very short period many times a minute. As a result, a continual stream of air interspersed with occasional beads of culture is drawn rapidly up the tube and normally travels through tap T_3 to the waste receiver *I*, where the culture drips into the lysol and the air is withdrawn through filter *J* into the vacuum source.

When a sample of the culture is required a tube is attached to the ground-glass cone of the sampling head *H*; tap T_4 is opened, to connect the tube to the negative air line, and tap T_3 is turned so that the effluent stream of air and culture is diverted from the waste receiver to the sampling head. The average time of transport of culture from growth vessel to sampling tube is less than 2 sec. The sampling tube can be surrounded by a thermos flask containing a suitable cooling or freezing mixture.

Safety switch

Most failures of the apparatus, other than breakages, lead only to a spoiled experiment. But if the suction pressure in the negative air line becomes inadequate while the apparatus is unattended the growth vessel may fill with culture, overflow through the liquid seal, and contaminate the hot room. To prevent that, manometer *K* acts as a mercury switch in the circuit of a pulsed motor (Fig. 4) connected to tap T_2 . When the suction falls below a safe limit the motor turns T_2 to the fully-off position, stopping the medium supply.

COMMENTS ON THE DESIGN AND OPERATING CONDITIONS

Air supply

Any sources of compressed air and vacuum are suitable if precautions are taken to prevent dangerously high or low pressures building up in the apparatus during faulty operation. The exact pressures and flow rates required in the air circuits depend mainly on the dimensions of the apparatus. In the prototype the positive air line supplied air at about 500 ml./min. and +5 cm. Hg pressure, and the negative line withdrew air at about 450 ml./min. and -10 cm. Hg pressure. Both lines were served by a dual purpose bellows pump driven by a $\frac{1}{8}$ h.p. induction motor geared down to 60 rev./min. The pumping unit

consisted of two flexible metal bellows connected to the common crankshaft by levers and connecting rods. The position of the fulcrum of each lever was separately adjustable, making the stroke lengths of the bellows independently variable. By this means it was easy to ensure that the volume of air blown into

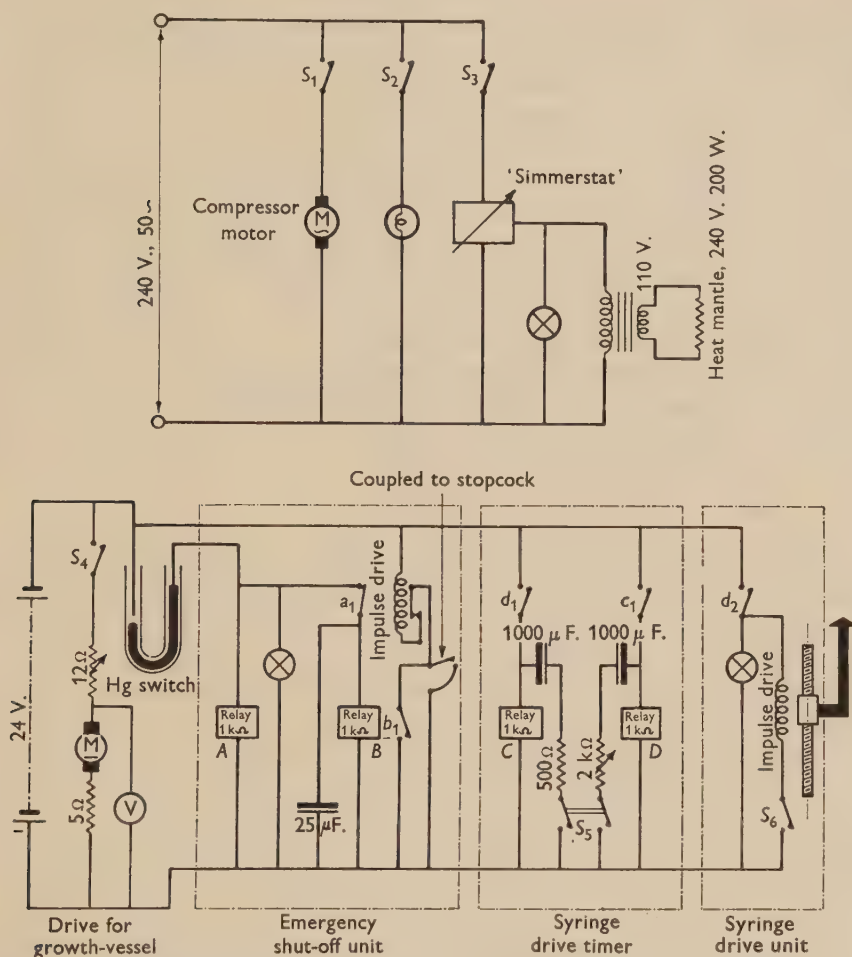


Fig. 4. The two electrical circuits associated with the apparatus, one operating at 230 V., A.C. and the other at 24 V., D.C.

the apparatus was always greater than the volume sucked out, so that air was never drawn into the growth vessel through filter *G*. The relative inefficiency of the ball-valves prevented the accidental build-up of very high or low pressures.

Medium supply

Silicone tubing (Dunlop Rubber Co. Ltd., Cambridge Street, Manchester, 1) was included in the medium flow lines as soon as it became available in order to increase the flexibility of the assembly. The silicone grease lubricating the

ground-glass joints made the apparatus rather difficult to clean, and it appears that its replacement by 'Teflon' sleeves (Loughborough Glass Co. Ltd., Loughborough, Leicestershire) may be a great improvement.

Air saturator

The usual method of humidifying air by bubbling it through water at room temperature is inefficient unless the bubbles are very small or their time of contact with the water very long. With rapid flow rates it is simplest partially to saturate the air at a high temperature and then to cool it so that condensation occurs. When the condenser is long enough the emergent air is 100 % saturated at ambient temperature. An air saturator is not absolutely essential, but it is advisable when very low growth rates are contemplated. For reasons discussed later the air-flow through the apparatus is considerably faster than is needed for adequate aeration. At 35° an increase in saturation from 50 to 100 % involves the removal of *c.* 1 ml. water/hr. for each l. air/min. When unsaturated air is used the effective concentration of the incoming medium will be greater than its made-up concentration and suitable corrections must be applied. In addition, unsaturated air will remove water from trap *L* and thereby alter the rate at which medium flows to the growth vessel. This effect is discussed in the next section.

Constant-head vessel

When a wide range of flow rates is required a constant-head variable-resistance system has practical advantages over one with a variable-head and constant-resistance.

In the prototype medium flows under a head of *c.* 30 cm. water, which is the sum of the depth of immersion of the tip of the bubbling tube in trap *L*, and the vertical distance between the tip of the bubbling tube in *B* and the tip of the dropping-jet in *E* (assuming that the density of the medium is unity). The contribution of air pressure to the total head may be increased at will by increasing the depth of immersion of the bubbling tube in *L*. Whatever the depth of water in *L*, in the absence of an air-saturator the apparatus must include some device for keeping it topped-up to constant level, or the medium flow rate will change progressively during operation.

It may sometimes be desirable to add a special metabolite at constant concentration over an extended period to a culture which has attained constant population density in its absence. This is done by means of the pre-mixing device without contaminating the main medium reservoir and without altering the culture dilution rate. The high-speed pulser is first used to inject into *B* enough metabolite to raise the concentration to the desired level. The pulser is then switched to variable speed and set at whatever rate is necessary, relative to the culture dilution rate, in order to keep the concentration constant. At the same time some of the metabolite may be added directly to the growth vessel through the inoculation port. Tap *T*₁ provides a constriction in the medium line which prevents the special metabolite from diffusing into the main reservoir against the stream of medium. At the end of an addition

period the pulser is switched off, *B* is drained through *T*₁ and allowed to refill at once with fresh medium.

As an improvement, the inlet capillary from *C* is led into *B* towards the bottom of the vessel, instead of at the top, to avoid the possible disturbing effect of suction on the motion of the syringe plunger. In addition, the capillary is provided with a 3-way tap so that the syringe can be refilled without disconnecting it.

Variable flow resistance

The grooved tap *T*₂ gave satisfactory control in the prototype at the faster dilution rates, provided it was mounted with the wide groove uppermost (Fig. 5) to prevent small air bubbles forming partial blockages. A great improvement is the substitution for *T*₂ of an array of six precision-bore capillary tubes ('Veridia' from Chance Bros. Ltd., Smethwick 40, Birmingham), mounted in parallel, giving flow-rates in the ratios 1:2:4:8:16:32. By means of taps the capillaries can be included in the medium circuit, individually or in all combinations, giving a range of 63 different dilution times between 20 min. and 21 hr. For this purpose long wide capillaries are preferred to short fine ones, being less likely to become clogged. The longer tubes are coiled to save space.

The efficacy of any type of flow resistance depends on the viscosity of the medium: the viscosity of water varies with temperature by nearly 2% per degree in the vicinity of 35°. It is therefore important that the temperature of the medium supply system be kept constant, and medium used for replenishing the reservoir should always be brought to hot room temperature before addition.

The growth vessel

When the vessel is spun too fast the culture leaves the bottom and clings to the sides in a smooth band. For best aeration and mixing conditions the speed used should be the highest at which a layer of culture remains always on the bottom of the vessel. In the prototype about 300 rev./min. was used, but larger vessels must be rotated at slower speeds.

The volume of cultures contained in the growth vessel diminishes slightly as the dilution rate is decreased. Since it is impracticable to measure the volume of culture contained in the vessel while it is spinning, dilution rates are determined accurately by injecting into the vessel ³²P (as phosphate) and measuring the radioactivity of samples collected at intervals. The straight line plot of log. radioactivity against time has a slope corresponding to the dilution rate. This procedure also eliminates errors which might arise through changes in the size of drop delivered by the jet in *E*, due to different media having different surface tensions.

One of the main disadvantages of using this type of growth vessel is that the design cannot be 'scaled-up' many times without decreasing the efficiency of aeration. But an alternative vessel of 2 l. capacity accommodating 400 ml. of culture has been included in a later model for use at low growth rates.

Culture disposal

It is theoretically possible to keep the culture volume constant indirectly by some device such as a double bellows pump which removes culture at the same rate as it adds medium. But it is practically impossible to ensure that the two pumping rates are absolutely identical, and any difference between them will have a cumulative effect. The volume of culture in the growth vessel, and therefore its growth rate, will then progressively increase or decrease throughout a run.

Electrical equipment

The use of 24 V., D.C. components in many of the circuits was dictated by the economic need to use cheap war-surplus equipment. But the current consumption at that voltage is inconveniently high, and the use of components with a much higher voltage rating is to be preferred.

FACTORS WHICH GOVERN THE DIMENSIONS OF THE APPARATUS

The identity of the organism, the temperature at which it is to be grown and the proportional composition of the medium (which may be simple or complex, and with or without a known limiting nutrient) having been decided, it is then necessary to determine three properties of the system: (1) γ_m , the maximum growth rate of the organism in the presence of an excess concentration of medium, expressed as doublings of dry wt./hr.; (2) Y , the dry wt. yield of organism per unit mass of nutrient consumed; (3) the curve relating growth rate and medium concentration, which will usually be of the general form shown in Fig. 6. A detailed and lucid discussion of the best way to determine these properties in batch culture appears in the publications of Monod (1942, 1949) which should be read by anyone contemplating studies on bacterial growth. There is now evidence, good in the case of *Aerobacter cloacae* (Herbert *et al.* 1956) and less convincing in the case of *Escherichia coli* (Perret, unpublished) that under some conditions Y may decrease at growth rates near to γ_m . But it is assumed here that, at a fixed temperature, Y is a constant independent of growth rate. It is also assumed that the relationship between growth rate and nutrient concentration depicted in Fig. 6 is independent of the previous history of the organisms, although theoretical and experimental evidence show that the assumption cannot be true. In fact, the curve in Fig. 6 strictly holds only for bacteria which are being subjected to changes of nutrient concentration at an infinitely slow rate, so that some of the published mathematical theory of continuous culture is misleading; this may have an important bearing on practical attempts to maintain bacteria at very low growth rates. It is hoped to discuss this discrepancy at length elsewhere, but for the present purpose of providing rough practical guidance in design the assumptions made are good enough.

Medium concentration

For metabolic studies it is advisable that the medium concentration, and therefore the amount of organisms/unit volume, should be as low as possible. The reasons for this appear later.

Let γ_1 be the fastest growth rate which it is desired to attain in the apparatus ($\gamma_1 < \gamma_M$) and let W mg./ml. be the lowest concentration of bacteria grown at

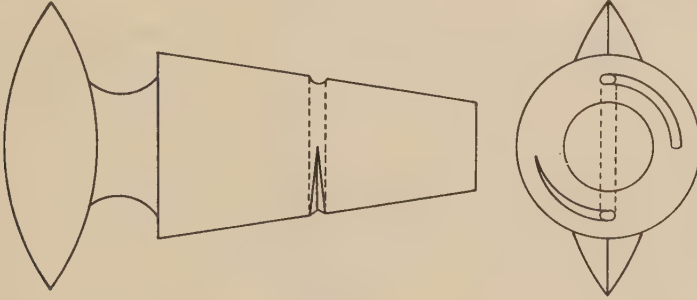


Fig. 5

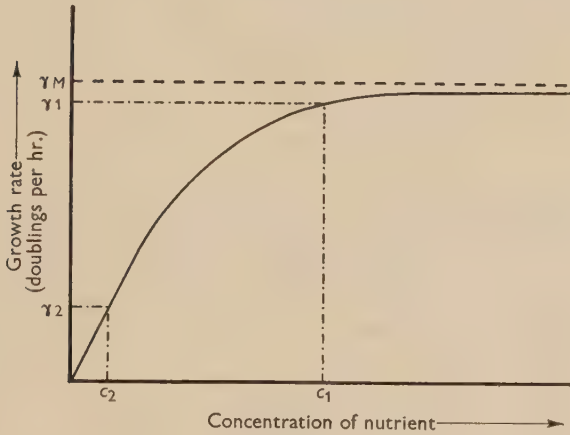


Fig. 6

Fig. 5. Details of the grooved taps used in the prototype. For satisfactory control of flowing liquids the taps must be mounted with the wide groove uppermost, so that air bubbles entering from below are not trapped.

Fig. 6. A portion of the type of curve relating growth rate and nutrient concentration. For most limiting nutrients the curve has the same general form, but the actual nutrient concentration scale may vary over a many thousand-fold range. γ_M is the maximum growth rate attained in the presence of an excess concentration of nutrient.

γ_1 , which will be acceptable for the experiments planned. The concentration of unconsumed nutrient in the effluent culture at γ_1 will be the same as c_1 , the ambient concentration in the growth vessel (Fig. 6). C , the lowest acceptable concentration of the incoming medium is therefore fixed by the equation

$$C = \frac{W}{Y} + c_1. \quad (1)$$

Culture volume

In general, the larger the culture volume the better. For economy in medium consumption, however, it may be necessary to decide how small the capacity of the growth vessel can safely be made. No precise answer is possible: it is ultimately a matter of opinion. But by making a few simple calculations it can at least become a matter of enlightened opinion. The minimum volume of culture is set by practical rather than theoretical factors. In order to avoid growth of bacteria up the medium inflow tube into the main reservoir it is necessary either to break the liquid continuity between sterile medium and culture, or always to have an inflow velocity so great that the bacteria cannot possibly migrate against the stream. A continuous jet of medium crossing an air gap provides the ideal solution, but it entails very high flow rates and correspondingly large culture volumes. The usual solution is to add the medium in discrete drops at regular intervals. Such discontinuous addition is in principle unsound, since it must lead to fluctuations of nutrient concentration in the culture. The higher the frequency of the concentration swing, and the lower its amplitude, the nearer the system approaches to its ideal state. Our knowledge is at present inadequate to prove that a given type of fluctuation is of negligible significance; one can merely say that it appears unlikely to be important in a particular context. Each worker must therefore decide for himself what fluctuations he is prepared to regard as unimportant for his purpose.

It can be seen from Fig. 6 that concentration changes have their greatest effect at the lowest growth rates. If γ_2 is the lowest growth rate to be attained in the apparatus and c_2 the corresponding ambient nutrient concentration, let it be decided that the increase in nutrient concentration following the addition of one drop of medium must not be greater than αc_2 , and that the frequency of the fluctuation must not be less than β/hr . Then if v is the volume of a single drop of medium

$$V_1 = \frac{vC}{\alpha c_2}, \quad (2)$$

and
$$V_2 = \frac{v\beta}{\log_e 2 \cdot \gamma_2}, \quad (3)$$

where V_1 and V_2 are respectively the lowest culture volumes to fulfil the fluctuation amplitude and frequency provisos.

A third factor which affects the culture volume is the size of sample necessary for the proposed investigations. If the volume of a sample is a very small proportion of the volume of culture in the growth vessel it may be removed directly without seriously upsetting the system. But if the sample volume is of relatively significant size it must be obtained by collecting the effluent culture continuously being withdrawn through the constant volume control tube. The time required for collecting a sample of given volume will then be proportional to the dilution rate. When the constancy of conditions in the growth vessel is not in question the composition of a sample will be the same, whether it be

obtained quickly or slowly. But when the constancy of conditions is in question, samples must be collected over a period short enough to reveal possible trends or cyclical variations. If the volume of a sample must be x ml. and the maximum time to be allowed for collecting it is t hr. when the culture is at its minimum growth rate γ_2 , then the volume of culture in the growth vessel must not be less than V_3

$$V_3 = \frac{x}{\log_e 2 \cdot \gamma_2 t}. \quad (4)$$

To satisfy all the conditions the volume of culture in the growth vessel must be at least equal to whichever is the largest of V_1 , V_2 and V_3 . As an example consider the case of *Escherichia coli* which is to be grown on a glucose-limited defined medium at a minimum density W of 1 mg./ml., a maximum growth rate γ_1 of 1.25 (in population doublings per hr.) and a minimum growth rate γ_2 of 0.2. Let the highest acceptable concentration fluctuation be arbitrarily fixed as $0.5 c_2$ at a lowest frequency of 300/hr. The size of a drop of medium is 0.05 ml., and it is required to collect 2 ml. samples in not more than 0.15 hr. From the published results of Monod (1942), $Y=c$. 0.23, $c_1=c$. 0.04 mg./ml. and $c_2=c$. 0.002 mg./ml. Then from equations (1)–(4): $C=4.4$ mg./ml., $V_1=220$ ml., $V_2=108$ ml. and $V_3=96$ ml., in which case the overriding value for the culture volumes is V_1 at 220 ml.

Medium, culture and air flow rates

The volume of culture, V , having been decided, then the maximum flow rate F_1 at which medium must be delivered to the growth vessel is easily calculated

$$F_1 = \gamma_1 V \log_e 2 \text{ ml./hr.}, \quad (5)$$

and the relationships between constant head and flow-resistance follow from Poiseuille's Law.

The constant-volume control system must obviously be able to remove culture at not less than F_1 ml./hr. But at high growth rates, if that minimum value is chosen, the sampling tube will be almost continuously full of culture moving at a comparatively slow rate, under anaerobic conditions, towards the sampling head. The effluent culture may then undergo such changes that samples no longer closely represent the composition of the main culture. The extent of the changes will depend on the density of the culture and on the time spent in transit. Since the culture density has already been set as low as possible in order to keep V small, it only remains possible to shorten transit time. This can be done by making the sampling tube of the narrowest capillary not likely to become blocked, and using a suction pressure at which the potential flow-rate of culture is much greater than F_1 . Droplets of effluent culture will then be swept rapidly to the sampling head by a high velocity air stream. At 35° water is about 40 times as viscous as air; so for a potential culture extraction rate of nF_1 , sterile air must be supplied to the apparatus at a rate of about $40 nF_1$ if negative pressure is not to develop in the growth vessel at low growth rates.

The author is considerably indebted to the staff of the Engineering Division of the National Institute for Medical Research, and to Mr N. Schunmann, for their expert and patient assistance during the construction of the original apparatus.

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A SYMPOSIUM ON PLANKTON

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The Plankton around the British Isles

By K. M. RAE

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From the distribution of certain planktonic forms around the British Isles, it is possible to recognize differences in the environment which are not readily detected by conventional hydrographic techniques. These so termed 'biological differences' in the environment appear to have a profound effect on the fluctuations in commercial fisheries.

The first evidence that this might be the case was found by workers at Plymouth when there was a sudden and drastic change in the planktonic fauna in the English Channel between 1930 and 1931. *Sagitta elegans*, a chaetognath which is to be found in areas where Atlantic and North Sea waters mix, was replaced in plankton catches taken off Plymouth by *S. setosa*, a typical North Sea form. It was assumed that there had been a weakening of Atlantic pressure at the mouth of the Channel and that the boundary between the two species of *Sagitta* had consequently shifted down the Channel to the west. From about the same time there started a progressive decline in the number of young demersal fish in the area and the average age of the herring in the commercial catches became higher until the local fishery collapsed. There was, too, a decrease in the phosphate content of the water during the winter which also could be attributed to a weakening of the flow into the western end of the Channel from the Atlantic, and it was suggested that this had led to poorer productivity of phytoplankton and so less food available for the young fish (Kemp, 1938; Russell, 1939).

However, these observations assumed a different significance when Wilson (1951) showed that the two water types, the one supporting *Sagitta elegans* and the other supporting *S. setosa*, had distinctive biological properties even after they had been microfiltered. Whereas the larvae of certain benthic forms collected off Plymouth would grow normally when cultured in *S. elegans* water, in *S. setosa* water they became stunted and died. Wilson could find no consistent differences between the chemical or physical properties of the two water types but showed that additions of *S. elegans* water would make *S. setosa* water a favourable culture medium. Consequently, growth factors associated with *S. elegans* or the environment in which it is found might be suspected.

Over recent years an effort has been made to relate in more detail the effect of Atlantic water on fisheries in the North Sea through a widespread plankton survey around the British Isles. Using an automatic sampler, the Hardy Plankton Recorder, monthly observations have been made on a number of steamship routes crossing the North Sea and running into the Atlantic and the Norwegian Sea. In each year it has been possible to trace the movements of a series of planktonic indicator species as they move from the open Atlantic off the coast of Ireland in the spring, northwards around the Shetlands, to reach the North Sea by the autumn. Over the period 1948 to 1954, there has been an advance in the timing of this sequence amounting to about two months. The indicator species have first appeared off Ireland in April instead of June and off the north-east coast of Scotland in June instead of August. Over the same period, there has been a noticeable southerly shift in the areas of most productive catches during the autumn herring fishery off the Scottish coast (Glover, 1955); the highest catches were taken about 140 miles farther south in 1954 than in 1948. Such results conform to an hypothesis that the herring migrate towards their spawning grounds under the influence of the advancing front of Atlantic water. During the autumn months the indicator species appear to move southwards into the North Sea at about 2 to 3 miles per day. So, if they reach the fishing grounds two months earlier, then they will reach a position some 150 miles farther south by any given date. It can also be shown in a more general way that the succession of spawning activity of the herring right down the east coast of the British Isles roughly coincides both in space and time with the southern boundary of the indicator species as they are carried southwards.

It has also been possible to follow the west to east drift of another Atlantic form, *Metridia lucens*, after it has entered the North Sea in the autumn of each of 10 years. The speed with which it moves away from the British coast between November and January can be correlated closely with the contemporary winds. It so becomes possible from a knowledge of the winds to forecast the distribution of *M. lucens*, or of the water carrying it, in relation to the haddock spawning grounds just before the onset of spawning. Wind data and estimates of haddock broods are available for 33 years and these two variates also correlate closely. A simple explanation of their relationship would again lie in the assumption that some factor carried by the inflowing Atlantic waters makes the environment beneficial to the survival of the haddock fry.

It would appear, therefore, that the ecology of the North Sea and more

particularly the fortunes of the commercial fish stocks there may be conditioned by some undefined environmental factors carried into the area each year from the Atlantic. It seems unlikely that those gross physical and chemical properties of the sea water which are usually measured are involved. As a working hypothesis one might conjecture that some growth factor is operative; some substance which becomes sufficiently depleted in the North Sea and English Channel to be limiting at times of high productivity unless it is replenished from the Atlantic. It is suggested that bioassay techniques may provide the most promising approach towards determining what the relevant factors are.

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Planktonic Bacteria

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From the Chambers's *Technical Dictionary* the definition of 'Plankton' is as follows: 'Animals and plants floating in the waters of the seas, rivers, ponds, and lakes, as distinct from animals which are attached to, or crawl upon, the bottom; especially minute organisms and forms, possessing weak locomotor powers.' The broader aspects of the activities of bacteria in lake water in relation to the establishment of the conditions which determine their growth and their relationship with other forms of plankton has been the primary object of current work in the bacteriology department of the Freshwater Biological Association. Before considering the results on the distribution of bacteria it is important to consider the physical conditions which occur in lakes, in view of the probable effect of those conditions on the bacteria. During the late spring and early summer the temperature of the surface water of a lake rises owing to increased solar radiation, the resulting difference in density of the water gives rise to the formation of two distinct layers of water, the upper layer or 'epilimnion' and the lower layer or 'hypolimnion'. Under some conditions the temperature is approximately the same at all depths in the epilimnion; below this layer, in the transitional zone known as the 'thermocline', the temperature falls rapidly with increasing depth; and in the hypolimnion the temperature, which is considerably lower than that of the epilimnion, does not change appreciably with increasing depth. Under some conditions the temperature in the upper layer falls rapidly with increasing depth from the surface to the thermocline. During the winter months the stratification of the water in Windermere is destroyed by the cooling of the upper layer or by the circulatory motion set up by the action of strong winds across the surface of the lake. In Windermere this mixing may take some weeks, but in the more shallow lakes, e.g. Esthwaite the 'overturn' of the waters may take place during the first autumnal gale.

It is apparent that following the period when the temperature is uniform throughout the lake, the effect of warmer external conditions is to warm the water at all depths but decreasingly so at increasing depths. As circulation of the water gradually ceases, a time is reached when the temperature at the bottom remains constant to within a fraction of a degree and stratification is then established. The nature of the thermocline and the depth at which it occurs vary greatly, but in general the depth tends to increase as the summer advances and the ratio of the volume of epilimnion to volume of hypolimnion therefore increases.

When the layering has begun, the amount of dissolved oxygen becomes gradually less in the hypolimnion owing to the stagnation of the water and the increased activity of the bacterial population of the mud surface. The dissolved oxygen continues to be present in normal amounts in the circulating waters of

the epilimnion. The concentration of dissolved oxygen in the lakes under investigation appears to be related to depth, shallow lakes being depleted to a greater extent because of the increased surface area of the mud exposed to the overlying water, than in the case of lakes with greater depth. The nearer the bottom, i.e. the interface between mud and water, the greater is the depletion.

Rainfall is another important factor to be considered in relation to the bacteriology of water. Rain falling on the drainage basin surrounding a lake is the carrier of the material brought into the lake by inflowing rivers. These inflowing rivers can also affect the temperature gradient of the water profile of a lake, the temperature of the inflowing river determining the position of the flow-in of water, warm river water going to the surface layers of the lake, and cooler river water sinking to lower layers. This was clearly shown in 1955 in Windermere. During a period of very calm dry weather, a heavy rainstorm struck the district and the result was for the river water to slide in under the surface waters, giving high numbers of bacteria at various depths in the water column. The extent and direction of wind force plays a very large part in determining bacterial distribution because of the water movements created in the surface layers of large bodies of water, and even more so in shallow lakes where the deeper layers can be disturbed in midsummer thus creating an artificial overturn condition for brief periods.

In general one may conclude, having regard to the above-mentioned physical conditions, that the bacteria present at any time in the water of a lake may be considered in three categories: (a) those washed in from outside sources, of which a large proportion will ultimately perish and others which may find conditions suitable for growth; (b) indigenous bacteria capable of existence in a dilute nutrient solution, as represented by lake water, and able to use for growth low concentrations of available organic matter; (c) bacteria dependent on a solid surface for their proliferation and therefore found in connexion with soil particles, mud detritus and plankton. This group may be further divided into types with special hold-fast mechanisms such as stalks and which are found only under natural conditions, and others which adhere to surfaces by mucilaginous material, the types which grow on the sides of containers.

Work over the past few years has involved the routine weekly sampling of two lakes in the English Lake District, namely Windermere and Esthwaite, and also monthly samples from the surface waters of every lake in the English Lake District. The results present a composite picture of the numbers and types of bacteria in these lake waters throughout the seasons.

Several hundred cultures of bacteria were isolated from samples of water plated out on standard agar (Taylor, 1940), in the above work. In view of past experience no attempt was made to classify them by the usual biochemical methods; instead a routine study was made of their ability to grow in media containing either inorganic or organic nitrogen, and different carbon sources.

Since the complex chemical constitution of natural waters makes them unsuitable for studies on the essential substances required for bacterial growth it was decided to use distilled water as a base and to control the composition

of the medium by the addition of known amounts of pure chemicals. The principle used was to prepare large amounts of distilled water inoculated with lake water and to add to the bulk known quantities of mineral salts, nitrogen and phosphorous sources, and a carbon and energy source. In various experiments one or more constituents was omitted or the quantity added in different concentrations. Bottles (8 oz.) were filled with the 'water medium' and the activity of the bacteria measured by determining the amount of oxygen consumed after periods of incubation at 20°. In further experiments lake water was used as a base and the effect of the addition of nitrogen, phosphorus and available organic matter was determined by the same method.

Although the work is by no means complete, and further experiments are required to clarify effects due to seasonal changes in the phytoplankton and chemical composition of the lake water, it is believed that a general picture of the bacteriology of lake water can be drawn as follows. Normally the growth of bacteria in lake waters is limited by the availability of the supply of carbon and energy sources. During long rainless periods the plate counts of bacteria decrease steadily and only increase after rain. This increase is partly due to washed-in bacteria but also to multiplication of types which avail themselves of the washed-in organic matter. The water acts as a selective agent in determining the types of washed-in soil bacteria which survive in this medium; some of the most common soil bacteria are absent. Factors which affect the selection of different types of bacteria include the concentration of dissolved substances, particularly phosphorus, the availability of organic nitrogen for types which are unable to use inorganic nitrogen, the presence or absence of particulate matter necessary for certain types of bacteria which require a site for attachment.

A large proportion of the types of bacteria in water have very weak powers of resistance and are difficult or impossible to maintain on laboratory media. All types which survived isolation from plates of standard agar grew in a liquid medium containing peptone; a proportion of these grew in a defined medium containing ammonium salts, and a small proportion when nitrate was the nitrogen source. No culture was obtained which used nitrate but not ammonia. More cultures grew in defined media containing nitrate or ammonia when a low concentration of agar was added than in its absence, due, it is believed, more to the growth-promoting activity of the agar (? source of growth factor) than to the availability of a semi-solid surface. Bacteria isolated after rain were more active physiologically, in respect to the use of ammonia and nitrate, than those isolated before rain. Plate counts on differential media after heavy rain usually showed a much larger relative increase in bacteria which required organic nitrogen rather than inorganic.

Experiments with a distilled water base medium showed that a readily available carbon and energy source, e.g. glucose, was rapidly decomposed by a mixed flora of lake bacteria when inorganic nitrogen and phosphate were added in the absence of all cations other than the traces present as impurities in the chemicals. Sodium and magnesium did not increase bacterial activity but calcium and magnesium or a mixture of all four ions did, phosphates of

sodium and calcium were equally effective. When nitrogen and available organic matter were in excess of requirements the amount of the latter which was decomposed at any time was a function of the concentration of the available phosphate. Phosphate in lake waters is slowly regenerated and the added organic matter similarly decomposed, but the addition of phosphate markedly hastens the process. Response to amounts of phosphorus as low as $1\text{ }\mu\text{g./l.}$ can readily be detected. The most important finding was that the addition of small amounts of iron (as FeCl_3) immobilized phosphate for bacterial use. A concentration of 0.5 mg. Fe/l. was sufficient to prevent any decomposition of glucose in the presence of $8\text{ }\mu\text{g. P/l.}$ during incubation for 4 days; even one-tenth of this amount was sufficient to depress the activity by a half.

The application of this technique to lake waters shows that nitrogen is usually present in excess of requirements and that phosphate is either regenerated or sufficiently slowly available to bring about eventually the decomposition of added glucose. However, when waters from different lakes were treated with small amounts of phosphate and excess glucose, the amount of glucose decomposed (measured by the oxygen consumed) was appreciably different and in all instances appreciably lower than in a simple 'artificial water' based on distilled water. It is concluded that this phenomenon is similar to that which occurs in agricultural soils and that part of the added phosphorus becomes unavailable. There appears to be little doubt that the concentration of iron in natural waters plays an important part in phosphorus immobilization.

Relationships between bacteria and other planktonic organisms in water

In view of the neglected state of research on fundamental problems of the bacteriology of waters, it is not surprising that little is known about the association of bacteria with planktonic animals and plants. Some simple relationships between bacteria and algae have been established, but more intricate problems, particularly those concerning stimulation or repression of one or the other by means of products excreted from the organisms, remain to be investigated. The production of complex organic compounds by algae in waters otherwise deficient in available energy and carbon sources for bacterial growth must be a factor in establishing the population of bacteria in water. On the other hand, bacteria can decompose organic matter and release available nutrients for algal growth. Whilst it has been established that in the sea and in lakes a large population of bacteria is associated with phytoplankton there is no evidence to show that bacteria are capable of attacking living diatoms. The excretion of organic compounds and antibiotics by living algae has been suggested by different workers. Present knowledge on the association of bacteria and the zooplankton seems too limited to the consumption, often on a selective basis, of bacteria by particle-feeding animals, and to decomposition of dead animals by bacteria.

The numbers of bacteria in different samples of phytoplankton is very variable. The bacteria are almost entirely rods or coccoid-rods and appear in localized concentrations. Such are seen sometimes in or on the mucilaginous

capsule of some blue green algae, or unattached. Examination of *Asterionella* shows the presence of a bacterium similar to *Caulobacter* species attached by a stalk to the cell wall of the diatom. Although the moribund diatoms are observed to have this type of bacterium attached, there is no significant increase in plate counts of bacteria following the death of these diatoms in lake water. No appreciable increase in available organic matter can be detected by the biochemical oxygen demand test in waters which have developed very large growth of *Asterionella*. There is, however, a marked increase in the numbers of bacteria in lake water on the death of large populations of blue-green algae, the biochemical oxygen demand increasing considerably as compared with the value for the same lake water without blue-green algae. Certain protozoans epizoic on flatworms form vacuoles whose internal contents contain bacterial rods like those present in water. These are clearly demonstrated by use of the phase contrast microscope. The existence of symbiotic relationships between bacteria and plankton, under natural conditions, remains to be established.

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ABSTRACT OF

Some Factors Affecting the Sinking Rate of Diatoms

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The vertical distribution of the eggs of the marine copepod *Calanus* in the sea is anomalous in that they are found almost entirely in the top 50 m., whereas from their specific gravity one might expect them to be evenly distributed, at least down to 100 m. It is suggested that a possible cause for this may be found in the mucus-like substance secreted by diatoms. Experiments were made by dropping single *Calanus* eggs through columns of sea water and of cultures of various species of diatoms and phytoflagellates, and measuring the rate of fall. In filtered sea water and in cultures of flagellates the rate of fall was regular, but in cultures of diatoms it was very irregular. Sometimes the egg falls more slowly than in filtered water but often it comes to a complete stop, resuming the fall after a few seconds or even minutes. Such 'hold ups' are most frequent in diatom cultures shortly after they have been put into the experimental vessel and are suspended evenly in the water. The viscosity of diatom cultures is not appreciably higher than that of sea water.

Toxic Marine Flagellates; their Occurrence and Physiological Effects on Animals

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From time to time in marine, freshwater and brackish habitats water blooms, consisting of enormous local growths of algae or protozoa, occur. In the sea these are commonly known as 'red tides' or 'red water', but may be of any colour from red to brown, green, yellow or merely cloudy. These blooms may be quite harmless, but are capable of producing conditions leading to mass mortalities, which can involve fish, shellfish and other animals. The mortality may be due to actual toxins produced by the bloom, or to secondary conditions such as oxygen deficiency, hydrogen sulphide liberation as a result of decomposition, or to bacterial pollution. This paper is concerned only with marine algal flagellates which themselves produce toxins, and is, of necessity, a very limited account of these, as the literature on the subject is vast and cannot be covered in a communication of this type. Most of the information available concerns toxic flagellates which form components of 'red tides'.

World distribution of marine and brackish water 'red tides'

The map (Fig. 1) shows the world-wide distribution of 'red tides' where mortalities have been shown to occur. This is based mainly on the papers of Nightingale (1936), Brongersma-Sanders (1948) and Hayes & Austen (1951), with additional records from other sources. This distribution map may not be complete, but gives a good illustration of the cosmopolitan distribution of the phenomenon.

The first alga we should mention is the blue-green alga *Trichodesmium* which is not, of course, flagellate. This forms widespread and recurrent patches in the Philippines, East Indian Archipelago and along the east coast of South America. In the Red Sea and north-west Indian Ocean blooms are due to *Trichodesmium* and also to dinoflagellates. *Trichodesmium* grows in bundles and rafts of filaments, and when forming a bloom colours the sea red by floating in dense masses on or near the surface. This appears to show a very variable toxicity, and only occasional blooms give mortalities.

The only member of the Chloromonadineae of which we have any record in toxic 'red tides' is *Hornellia marina* (Subrahmanyam 1954), which occurs off the south-east and south-west coasts of India.

In brackish water the Chrysophycean flagellate *Prymnesium parvum* Carter (1937) has been reported as causing mortalities in Denmark (Otterstrøm & Nielsen, 1939) and more seriously in the Israeli fish ponds (Shilo & Aschner, 1953). This organism is always present in some quantity, but can suddenly bloom and become toxic, more or less overnight.

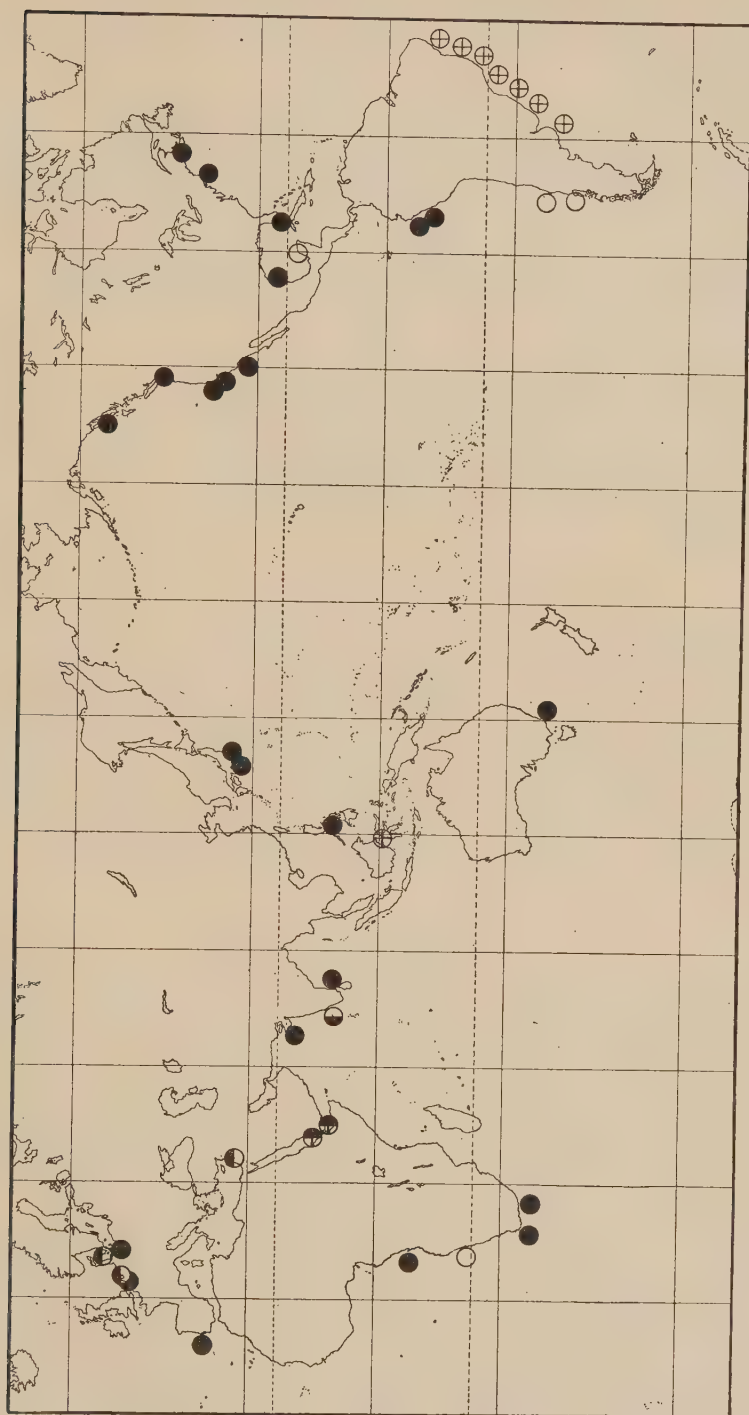


Fig. 1. Map showing the distribution of toxic 'red tides'. Water blooms are due to the following: ●, Dinoflagellatae; ⊙, Chrysophyceae; ⊕, Chloromonadineae; ⊙, Cyanophyceae; ⊕, Dinoflagellatae and Cyanophyceae; ○, toxic alga not known.

The remaining areas shaded on the map have blooms of dinoflagellates of various species, and the following is a list of some of the localities and the causative organism:

Southern California, U.S.A.: *Goniaulax polyedra* Stein (Kofoid, 1911);
G. catenella Whedon & Kofoid (1935).

Washington State, U.S.A.: *Gymnodinium splendens* Lebour (Nightingale, 1936).

Florida and Gulf of Mexico: *Gymnodinium brevis* Davis (1948, Wilson & Ray, 1956).

Bay of Fundy, Canada: *Goniaulax tamarensis* Lebour (Needler, 1949).

Belgium (Bruges-Zeebrugge canal): *Pyrodinium phoneus* Woloszynska & Conrad (1939).

Portugal: *Goniaulax polyedra* Stein (Santos-Pinto, 1949).

Angola, West Africa: *Exuviaella baltica* Lohmann (Silva, 1956).

Japan: *Gymnodinium mikimotoi* Miyake & Kominani (Miyajima, 1934);

Cochlodinium catenatum Okamura (Miyajima, 1934).

Sidney, Australia: *Goniaulax polyedra* Stein? (Whitelegge, 1891, but see also Kofoid, 1911).

Causes of 'red tides'

Blooms occur only when a number of parameters are simultaneously suitable. This subject is by no means understood as yet, but it is obvious that high concentrations of nutrients, particularly nitrate and phosphate, must be available, temperatures must be suitable and there must also be enough phytoplankton to take advantage of these conditions while they persist. These conditions can arise in a number of ways, but among those suggested are pollution, as in the Belgian canals, by freshwater outflows rich in nutrients or by upwelling of deep cold rich water, such as occurs along the west coast of Africa from the Benguela current, and probably also in many other 'red tide' areas.

Economic importance of mortalities

The economic importance obviously depends upon the area in which the mortality occurs. The stranding of large amounts of decaying fish near areas of dense population causes a serious disposal problem and, in addition, there is frequently an irritant gas associated with 'red tides' which causes considerable discomfort (Woodcock, 1948; Ingle, 1954). There is also the loss to commercial fishing in areas around India, Florida and South America, and the damage to the oyster and other shellfish industries, particularly in Japan and America.

On the other hand, a more serious human hazard arises in the problem of paralytic shellfish poisoning (particularly in North America and Japan). Certain species of *Gymnodinium* and *Goniaulax* (e.g. *Goniaulax catenella* and *G. tamarensis*) bloom in inshore waters and shellfish which feed on the flagellates may not be killed but concentrate the poison in their digestive glands. When these shellfish are eaten they can cause serious illness or death. This toxin is thermostable and survives cooking and even canning. It produces vomiting, facial paralysis, later extending to the limbs, and death in severe cases from respiratory paralysis (Dodgson, 1928).

A great deal of work has been done in America on the ecology of the species involved, the hydrographical conditions of the affected areas, and the pharmacology of paralytic shellfish poison, and it is beyond the scope of the present paper to do more than briefly mention this most important contribution. In view of the human problems involved the emphasis has been on the medical and preventive aspects. Extracts from the digestive glands of the poisoned mussels have been prepared and injected into mice, and by this means a bio-assay technique has been developed (Stephenson, Edwards, MacDonald & Pugsley, 1955). The poison has also been extracted from phytoplankton and the connexion between the two established (Sommer, Whedon, Kofoid & Stohler, 1937; Riegel, Stanger, Wikholm, Mold & Sommer, 1949). It is now possible by routine examination of phytoplankton samples to determine the times of year at which dangerous degrees of toxicity are likely to occur. Chemical analysis has also been attempted, and study of the pharmacological action of the toxin, in an effort to determine the exact composition of the toxin, but so far nothing has been published on this subject, though it is known that the compound is basic in nature. So far as we know no work has been done with bacteria-free cultures of toxic marine flagellates, but evidence at present indicates that the flagellates themselves produce the toxins.

Physiological effects of the toxins from marine algae

Our interest in the problem of red tides was stimulated by the discovery of a new species of dinoflagellate, *Gymnodinium veneficum*, which proved to be toxic. This species was isolated by Dr Mary Parke from the Plymouth area and described by one of the authors (Ballantine, 1956). The flagellate is about 12 μ . long, yellowish brown in colour and is a phototrophe; it produces a very powerful toxin, most of which is secreted into the water. No red tides occur near Plymouth, nor have there ever been any reports of fish mortalities or of poisonous shellfish due to this cause. It has therefore been possible to investigate the mode of action of the *G. veneficum* toxin without the emphasis of a problem in preventive medicine.

Experiments have been carried out with a wide range of animals, and all except polychaetes are affected to a greater or lesser extent. In particular small fish, mainly gobies, have been used as test animals; these die within 10 min. in toxic cultures. Death in fish seems to be due to some form of respiratory failure. The action of the toxin is certainly on the nervous system. The first symptoms are a complete loss of balance, combined with an intense change of colour pattern. This is followed by a period of violent gasping, and intermittent unco-ordinated bursts of movement. During the quiescent intervals, which become progressively longer, there is no response to sensory stimulation. At the point of death there is no sign of haemolysis in the blood, the heart is beating, and excised muscles respond to direct stimulation. In the case of the frog, injection of the toxin into dorsal lymph sac has an immediate paralysing effect. Sensory stimulation has no effect and breathing stops completely. Yet the frog may live in this paralysed state for a couple of days before dying; the necessary oxygen supply is provided by exchange through the skin.

The *Gymnodinium veneficum* toxin has been obtained salt-free by dialysis and concentrated by evaporation under reduced pressure. Its effects on isolated tissues from a variety of animals have been studied. Only a few conclusions can be mentioned here. The toxin acts by depolarizing excitable membranes, rendering them inexcitable. Both nerves and muscles are affected, but in the intact animal death occurs because of interference with transmission in the nervous system. The effect is reversible in isolated tissues, but the whole animal cannot dispose of the toxin. Isolated hearts from most animals can be inhibited by the toxin. *Buccinum undatum* (whelk) is particularly sensitive; the heart stops in systole and can be used for bioassay. Frog sartorius muscle

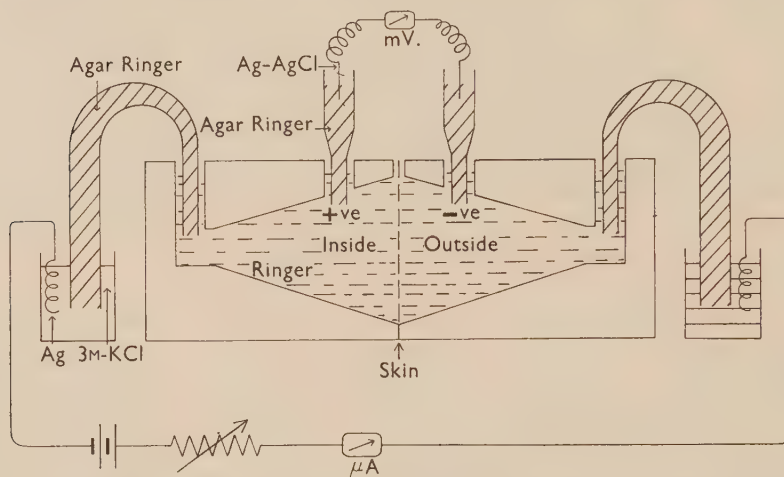


Fig. 2. Apparatus for measurement of potential and resistance of isolated frog skin (after Ussing; for explanation see text).

becomes inexcitable both to indirect (nerve) and to direct (muscle) stimulation. Membrane potentials were measured by inserting a micro-electrode into the muscle fibre; the resting potential of about 80 mV. drops to a very small value within a few minutes of application of the toxin, and excitability disappears.

This effect was studied in more detail in frog skin. Isolated skin, even with identical Ringer solution on both sides, shows a potential difference of about 50 mV. between the two sides, with the inside positive. This potential drops to zero within a few minutes of the addition of *Gymnodinium veneficum* toxin to the inside of the skin. The apparatus used in these experiments is shown in Fig. 2, and is adapted from that described by Koefoed-Johnsen, Ussing & Zerahn (1952). A circular piece of skin isolated from the abdominal surface of the frog separates two conical chambers cut in Perspex. Skin potential is recorded from the two electrodes near the skin, one on either side. Current can be forced through the skin from an external battery through the two outer silver electrodes via agar bridges. From the relation between the external current imposed and the resultant potential measured across the skin, a measure of the total skin resistance can be obtained.

The equivalent circuit of the skin, based on Ussing & Zerahn (1951), is shown in Fig. 3. A sodium pump in the skin forces sodium ions across the skin, and the result can be represented by a battery E_N in series with a resistance which opposes the flow of the sodium ions. The passive resistance of the skin to other ions acts as a resistance in parallel to the battery circuit. The thin lines show the meter for recording skin potential and also show the external circuit for imposing an extra current through the skin. When the imposed current is varied the recorded skin potential varies, and the slope of the current-

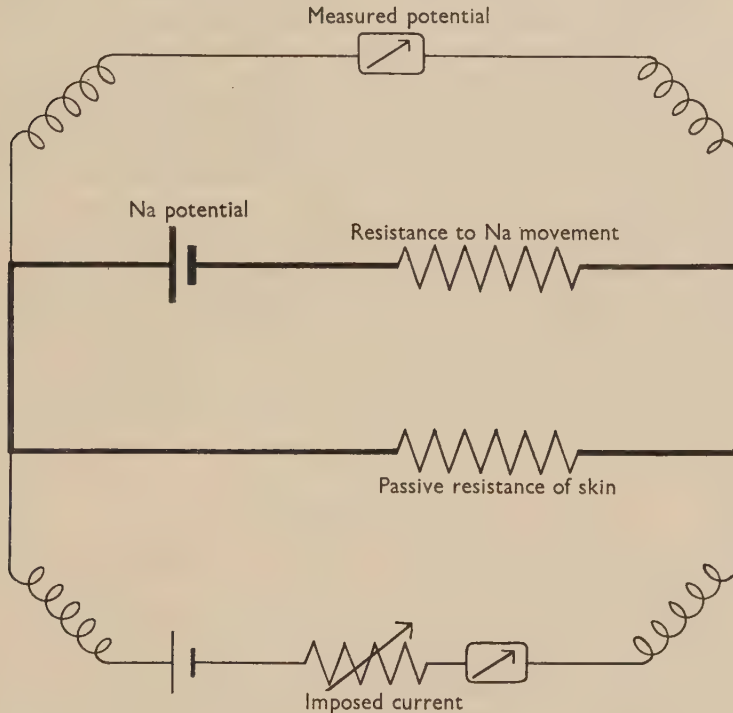


Fig. 3. Equivalent electrical circuit of frog skin (after Ussing; for explanation see text).

voltage relationship provides an indication of the total skin resistance offered (it actually gives the sum of the conductances of the two paths). From the equivalent circuit it can be seen that the skin potential could be destroyed either by inhibition of the sodium pump or by a very large decrease in the passive skin resistance—which would effectively short circuit the battery E_N . We have found that the skin resistance is completely unaltered by the addition of the *Gymnodinium veneficum* toxin. In contrast, destruction of the membrane potential by the addition of potassium chloride is associated with a 12-fold increase in the conductance of the skin.

From this and other results it is concluded that the flagellate toxin affects the exchange of sodium ions across cell membranes. It has not so far been possible to specify the main sites of action in a whole animal, whether in the central or

peripheral nervous system. Death certainly occurs before the skeletal muscle system has become inexcitable to direct stimulation. But in general we have found that well-sheathed nerves are less affected than muscle tissue, whereas exposed sensitive nerve endings are rapidly inhibited. Experiments on transmission through mammalian superior cervical ganglia have shown that ganglionic potentials can be depressed, with particular emphasis on the disappearance of the slow after-potential.

Studies by Kellaway (1935) and by Fingerman, Forester & Stover (1953) showed that the toxic extracts from 'livers' of poisonous shellfish act on the nervous systems of mammalian and amphibian animals. Fingerman *et al.* concluded that this toxin acted in a manner similar to curare, by preventing the response of skeletal muscles to acetylcholine.

The toxin from *Gymnodinium veneficum* is probably different from the shellfish poison. We have reported in more detail (Abbott & Ballantine, 1957) that extraction of the *G. veneficum* toxin under acid conditions, similar to those used for shellfish extraction, alter the action of the poison. But if the *G. veneficum* toxin is extracted under neutral conditions the effect on frog nerve-muscle preparations is very like that described by Fingerman *et al.*: loss of indirect excitability first, at which stage end-plate potentials can be recorded, followed by disappearance of the end-plate potentials and eventual loss of excitability in the motor nerve. But in the case of the *G. veneficum* toxin the effect is due to membrane depolarization rather than to a curare-like inhibition of response to acetylcholine.

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Utilization of Trace Elements by Marine Unicellular Algae

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The relationship between the supply of trace elements and the growth of phytoplankton can be considered from many different aspects. The supply of an essential trace element may limit the growth of the unicellular algae by limiting the growth rate, the total crop or both. The physical or chemical form in which the element is present may also be of importance in governing its availability to the organism. The mere demonstration of an absolute requirement for a particular trace element is a technically difficult matter by direct means. In recent years, however, the value of chelating agents in trace element studies has been pointed out (Hutner, Provasoli, Schatz & Haskins, 1950) and these workers and others have used chelating agents both for the qualitative demonstration of trace element requirements and for some quantitative measurements (Hutner, 1948; Walker, 1953; 1954). Considerable amounts of physico-chemical data are now available concerning the equilibria between certain chelating agents and free metal cations; such data enables these techniques to be much more fully exploited than hitherto. It is the purpose of this communication to describe some recent results which have been obtained during an investigation of the effects of ethylenediamine tetra-acetic acid (EDTA) on the growth of a marine unicellular alga and to discuss the possible implications of the results on various aspects of trace element utilization.

When EDTA is added to sea water a large number of equilibria are set up in which the cations present compete for the available EDTA. In addition, four proton association equilibria (corresponding to the stepwise association of a weak quadribasic acid) must be considered. A general formula has been developed for dealing with multi-equilibria systems of this type. This avoids manipulating the large numbers of simultaneous equations which are needed to deal quantitatively with such systems. In its simplest form the general formula is applicable to a system of any number of competing equilibria involving 1:1 chelate formation between metal and chelating agent. The formula is easily extended to include the formation of chelates of other structure.

By applying the general formula to solutions of various strengths of EDTA in sea water it is possible to calculate the concentrations of all the biologically important cations which can exist in equilibrium with the chelating agent. Because of the great stability of most chelates of EDTA and the very small amounts in sea water of many of the trace elements it is possible to produce culture media in which the concentration of the free ions of many metals is extremely low. This has been realized by the previously mentioned workers and it has become the usual practice to add relatively large amounts of trace

elements to such media to compensate for this effect. The composition of these media, however, is usually such that the addition of a certain quantity of a trace element causes a disproportionately small increase in the concentration of the free ions of that metal. Calculations show that the concentration of free ions of many metals in typical media of this type is of the order of 10^{-9} M and below.

That many of the unicellular algae will grow at optimum rates in medium of this composition raises interesting problems about the mechanism of utilization of trace elements from such equilibrium mixtures. Two possibilities suggest themselves. First, it is possible that the chelated metal is made available as a result of the organic moiety of the complex being metabolized and the metal being liberated in ionic form. Alternatively, it is possible that the organisms are capable of mobilizing the very low concentrations of free ions the concentration of which will tend to be maintained by some of the complex dissociating to restore the equilibrium. The systems set up in EDTA medium are capable of experimental investigation. Either one can examine the effect of chelation on the toxicity of a heavy metal ion or, alternatively, the effects of chelation on the growth-promoting activity of an essential trace element can be considered. In either case the biological activity might be expected to be related to the concentration of the particular form or forms in which it is available to the organism.

The results reported have been obtained using bacteria-free cultures of a marine unicellular alga *Phaeodactylum tricornutum*. Culture methods and measurements of growth, with certain minor modifications to be described elsewhere, have been by the techniques previously developed for use with this organism (Spencer, 1954). The cupric ion is increasingly toxic to the test organism at concentrations in excess of about 2×10^{-6} M. The Cu-EDTA complex does not become toxic until concentrations in excess of 5×10^{-3} M are reached. Calculation shows that the toxicity of the cupric chelate is probably accounted for by the equilibrium concentrations of cupric ions which, under these conditions, are of the order required to cause a comparable growth-inhibition in the absence of EDTA.

Similar results are obtained when the effects of EDTA on the availability of manganese are investigated. By prolonged cultivation at high EDTA concentrations it is possible to obtain an inhibition of growth which is reversed by the addition of manganese. Under these conditions the growth-promoting activity of additions of manganese can be shown to be strictly related to the concentration of ionic manganese and to be independent of the total manganese concentration or the concentration of Mn-EDTA complex. These results are in accord with the generally accepted fact that EDTA is not readily metabolized.

The results further indicate that the organisms must be capable of mobilizing manganese ions at an optimum rate at a concentration of about 2×10^{-9} M-Mn⁺⁺. The results with Cu-EDTA complex suggest that chelated copper is similarly biologically inactive. The concentration of cupric ions existing in the medium used for the manganese studies can be shown to be of the order of 10^{-14} M. Copper is generally supposed to be an essential trace element for growth and

yet no evidence of any copper-deficiency symptoms has been obtained even after several months of cultivation under these conditions. Similar considerations apply to other trace elements, particularly zinc and cobalt. The concentrations of these elements in ionic form in the medium used is of the order of 10^{-13} M and 10^{-14} M, respectively. Evidently a 'trace element free' sea water will have to be prepared before the utilization of these elements can be investigated in the same way as manganese.

By analogy with the results obtained with manganese it seems reasonable to expect that the utilization of copper, zinc and cobalt from such equilibrium mixtures will follow a similar pattern. A strong suggestion that the critical concentration for the mobilization of these ions at optimum rate may well be below the concentrations obtained in the present studies can be seen from the results reported by Walker (1953). This worker was apparently able to obtain optimum growth of *Chlorella* in an EDTA medium which had been prepared by techniques which seem to preclude chance contamination with appreciable quantities of trace elements. It can be shown that the ionic concentrations in Walker's complete medium were of the order of 10^{-9} M- Mn^{++} ; 10^{-11} M- Zn^{++} ; 10^{-14} M- Cu^{++} . Inhibition of growth only occurred when the concentration of one or other of these ions was lowered by decreasing the total concentration of the particular metal.

It therefore begins to appear that at least some of the unicellular algae may be able to mobilize their essential trace elements from very low ionic concentrations. This possibility may have important implications when we consider various aspects of trace element utilization and their possible role as a regulating factor of the growth of phytoplankton in nature.

The problem of the supply of metals when present as sparingly soluble hydroxides has troubled algologists for many years. The amount of iron which can exist in true solution in sea water at pH 8 is exceedingly small and apparently cannot exceed a concentration of *c.* 10^{-14} M (Cooper, 1937). The usual practice is to add iron to culture media in complex form in order to prevent the precipitation of ferric hydroxide. Many marine algae can at least partially satisfy their iron requirements when an iron compound is supplied to them largely in some particulate form; it has been suggested that direct utilization of particulate ferric hydroxide can in some cases occur (Harvey, 1937). Nevertheless, even in these cases the marked superiority as an iron source of complex soluble forms can be demonstrated. It is obvious that any complex of iron which will prevent the precipitation of ferric hydroxide must maintain in solution a lower concentration of ferric ions than exists in equilibrium with solid ferric hydroxide. It is beginning to appear increasingly unlikely that the ferric-EDTA chelate will be metabolized. A suggestion has recently been made that even the ferric citrate complex may not be metabolized (Goldberg, 1952). In medium containing iron in complex form, it therefore seems most likely that, as in the case of manganese, the organisms mobilize the ferric ions. The superiority of complexes as an iron source might therefore be due to the fact that the adjustment of the equilibrium subsequent to the removal of ions from such a system is instantaneous. This contrasts to the finite time required for

the corresponding adjustment to occur by solution of a solid phase. Similar considerations may of course apply to the observed beneficial effects on the availability of trace elements for higher plants when chelating agents are added to soil.

If we consider the trace element supply to the phytoplankton in nature, a certain analogy with the position in EDTA medium is apparent. In both cases the organisms are presented with a large total amount of a particular trace element but at a low immediately available concentration. Except in the case of self-contained bodies of water or perhaps in conjunction with high concentrations of nitrogen and phosphorus when very heavy growths may occur, it seems unlikely that the growth of the phytoplankton will seriously decrease the total amount of a particular trace element. With the more usual relatively sparse crops it has always seemed that the most likely way in which a trace element deficiency might occur in nature was by the concentration of a particular element becoming decreased below the concentration at which it can be mobilized at an optimum rate. The very low concentrations at which this would become important, at least with some organisms, therefore seems to suggest that trace elements are unlikely to have such a regulating effect. The results emphasize even more the generally recognized importance of the form in which the main store of trace elements is present and in particular emphasize the importance of the nature of the equilibrium between the store and the supply of ions.

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Auxotrophy and Organic Compounds in the Nutrition of Marine Phytoplankton

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The artificial cultivation of marine planktonic diatoms dates from the studies of E. J. Allen at Plymouth, who maintained 'persistent' (i.e. 'unialgal', or 'bacterized') cultures of several species in enriched natural sea water (Allen & Nelson, 1910; Allen, 1914). Defined media failed to support the diatoms, but a very small addition of natural sea water or algal extract sufficed to make good the deficiency. Allen inferred the presence in sea water of an organic substance active in catalytic amounts, which he likened to the then recently discovered 'vitamine' from the husk of rice. One result of Allen's studies was the recognition that sea waters of different origin varied greatly in their ability to promote the growth of algae. Trace metals such as manganese and iron were involved but, as Harvey (1939) showed, they could by no means always account for all the observed effects, since certain compounds containing divalent sulphur were needed to complete the enrichment of infertile water. Experiments on settling of pelagic larvae were later to lead Wilson (1951) to similar thoughts concerning the biological activity of different waters and to conclude that the activity—in this case rendering a substratum suitable for settlement—was due chiefly to the presence of living bacteria (Wilson, 1954).

Allen's invocation of what is now called auxotrophy was out of harmony with contemporary views on algal nutrition, for the work of Molisch (1895, 1896), Benecke (1898) and Chodat (1913) had given rise to the belief that algae were by and large autotrophic like higher plants (though facultative chemotrophs were known). Furthermore, the earliest critical study with an axenic culture of a marine diatom (Peach & Drummond, 1924) was on the supralittoral *Phaeodactylum tricornutum* (syn. *Nitzschia closterium* forma *minutissima*) which, contrary to the rule, as it has since proved, grows on the simplest mineral media. The subsequent wide use of this diatom in physiological studies has tended to obscure the import of Allen and Harvey's observations. Meanwhile, a new generation of algal physiologists was coming to the conclusion that algae were not as exclusively autotrophic as had been thought, and that the error had been due to a selection of autotrophs by the isolation methods previously in use (Pringsheim, 1946). The introduction of the use of soil extract by Pringsheim (1912, 1926) greatly increased the range of organisms which it was possible to grow in axenic culture, with the result that the extent to which auxotrophy is prevalent among the lower groups of algae gradually became evident (see Provasoli & Pintner, 1953). The culture medium familiar to marine biologists by the name of 'Erdschreiber' (Føyn, 1934) owes its success largely to the presence of soil extract.

A first step to an understanding of the government of a micro-organism in the field is an analysis of the properties of any natural substances which are required to support axenic growth *in vitro*. The benefit of soil extract, for example (or indeed natural sea water), might in any instance be derived from any or all of a number of properties (Pringsheim, 1935), among which may be listed: (a) control of trace metals; (b) pH buffering; (c) supply of organic micronutrients (satisfaction of auxotrophic requirements); (d) other quasi-physical properties such as poisoning or neutralizing of toxicity. Wilson's observations on the effect of bacteria on the settlement of *Ophelia* have been referred to; they parallel the experiences, too numerous to instance, of those who have attempted to cultivate fastidious algae. The difficulty of establishing axenic cultures of members of the phytoplankton is evidence of a dependence upon bacterial products (the problem of actual purification usually not being great). This, however, does not necessarily indicate auxotrophy on the part of the alga, since bacterial products may have any of the properties listed above for soil extract; properties which under given circumstances would cause them to influence algal growth.

Culture solutions with trace metals nicely balanced against the metal chelator ethylenediamine tetra-acetic acid (EDTA; Hutner *et al.* 1950; Provasoli & Pintner, 1953; Droop, 1955*a*) and containing tris-(hydroxymethyl)-amino-methane (TRIS) as a pH buffer (Provasoli, McLaughlin & Pintner, 1954; Droop, 1955*b*) have effectively replaced soil extract so far as these functions are concerned, and have facilitated the cataloguing of vitamin requirements of a number of organisms previously dependent upon soil or other extracts. Thiamine and particularly cobalamin (vitamin B₁₂) are the B vitamins most frequently cited as being required by algal flagellates and diatoms. In many cases soil extract and natural sea water can be completely replaced by these two vitamins, appropriate cation mixtures and a pH buffer. Biotin has been recorded among the requirements of the freshwater *Ochromonas malhamensis* (Hutner, Provasoli & Filfus, 1953). It appears that heterotrophy other than auxotrophy is rare among the few marine pigmented species studied nutritionally. *Hemiselmis virescens* is an example of an alga dependent upon amino nitrogen (preferably glycine), while some others are facultative in this respect (Droop, 1955*a*). All, including those which are phagotrophic, appear to be obligate phototrophs (at pH 8 at any rate). The colourless species of course are necessarily chemotrophic and the non-phagotrophs among them (e.g. *Peridinium depressum*) require dissolved organic compounds capable of providing energy. None of the latter group has yet been cultivated free of bacteria.

The identification of thiamine as one of the active principles in soil extract was made by Lwoff & Lederer (1935); since then a need for thiamine has been recorded in a number of Chlorophyta, Euglenophyta, Pyrrhophyta and Chrysophyta (cf. Provasoli & Pintner, 1953). Marine species include several chrysomonads (Droop, 1954), but the effect of thiamine for them was merely stimulatory. In point of fact, an absolute requirement for thiamine in the part of any marine phytoplankton has yet to be established.

The situation with vitamin B₁₂ is less ambiguous since the requirement, when present, is absolute and demonstrable by a clear dose/response relation. The isolation of the crystalline vitamin from ox liver (Smith, 1948) was quickly followed by its identification by Hutner and his co-workers with factors needed by the flagellates *Euglena gracilis* and *Ochromonas malhamensis* (Hutner *et al.* 1949, 1953). This led to recognition of a need for vitamin B₁₂ on the part of an impressive number of marine and freshwater algae and flagellates which had previously been cultivated only with the aid of soil, liver or other natural extracts (Provasoli & Pintner, 1953; R. A. Lewin, 1954; Droop, 1954, 1955*a, b*; Sweeney, 1954). Soil extract may contain considerable amounts of vitamin B₁₂: for example, 1–8 µg./l. cold water extract (Robbins, Hervey & Stebbins, 1950) or 9–60 µg./l. of hot alkaline extract (Droop, unpublished). Vitamin B₁₂ requirers represent a large proportion of the marine organisms which have so far been grown in axenic culture. Tables 1–3 are not by any means representative of the phytoplankton, either oceanic or neritic, but they do contain some pelagic species, and an interest by marine ecologists in the distribution of vitamin B₁₂ seems to be well justified.

Table 1. *Marine algae known to require vitamin B₁₂*

Dinophyceae	
<i>Amphidinium klebsii</i>	Provasoli, unpublished
<i>A. rhynococephalum</i>	Provasoli, unpublished
* <i>Exuviaella cassubica</i>	Provasoli, unpublished
* <i>Gymnodinium splendens</i>	Sweeney, 1954
<i>Gyrodinium californicum</i>	Provasoli, unpublished
<i>Peridinium balticum</i>	Provasoli, unpublished
<i>P. chattoni</i>	Provasoli, unpublished
* <i>Prorocentrum micans</i>	Droop, unpublished
Cryptophyceae	
<i>Hemiselmis virescens</i>	Droop, 1955 <i>a</i>
Chrysophyceae	
* <i>Isochrysis galbana</i>	Provasoli, unpublished
<i>Microglena arenicola</i>	Droop, 1955 <i>a</i>
<i>Monochrysis lutheri</i>	Droop, 1954, 1955 <i>c</i>
<i>Prymnesium parvum</i>	Droop, 1954
<i>Syracosphaera carterae</i>	Provasoli & Pintner, 1953
<i>S. elongata</i>	Droop, 1954
Bacillariophyceae	
<i>Amphora perpusilla</i>	Hutner & Provasoli, 1953
* <i>Skeletonema costatum</i>	Droop, 1955 <i>b</i>
Chlorophyceae	
<i>Brachiomonas submarina</i>	Hutner, unpublished
<i>Stichococcus</i> sp.	Lewin, 1954
Cyanophyceae	
<i>Phormidium persicinum</i>	Provasoli & Pintner, 1954

* Pelagic species.

The numerous analogues of vitamin B₁₂, of varying activity for different organisms (Kon, 1955; Ford & Hutner, 1955), also deserve the attention of ecologists, in so far as these analogues may be shown to occur naturally and to evince a response in any organism. The pattern of specificity of several species

represented in Table 1 was studied by L. Provasoli (Haskins Laboratories, New York) and myself with the aid of vitamin B₁₂-like compounds isolated by E. S. Holdsworth and J. E. Ford of the National Institute for Research in Dairying, Shinfield, near Reading, Berkshire (Table 4). It appears that one or other of the specificity patterns known in other organisms is shown by most of these marine algae. Of the fourteen species tested, eight responded only to the

Table 2. *Some marine algae known not to require vitamin B₁₂*

Cryptophyceae	
* <i>Rhodomonas lens</i>	Provasoli, unpublished
Chrysophyceae	
<i>Stichochrysis</i> sp.	Hutner, unpublished
Bacillariophyceae	
<i>Phaeodactylum tricornutum</i>	Peach & Drummond, 1924
Chlorophyceae	
<i>Dunaliella</i> spp.	Hutner, unpublished
<i>Nannochloris oculata</i>	Droop, 1955a
<i>Prasiola stipitata</i>	Lewin, 1955
Phaeophyceae	
<i>Ectocarpus parasitica</i>	Hutner, unpublished
<i>Waerniella lucifuga</i>	Droop, unpublished
Rhodophyceae	
<i>Porphyridium cruentum</i>	

* Pelagic species.

Table 3. *Some marine algae in axenic culture for which data are wanting*

Dinophyceae	
<i>Glenodinium foliacium</i>	Droop, unpublished
* <i>Peridinium trochoideum</i>	Droop, unpublished
<i>Ocyrrhis marina</i>	Droop, unpublished
Chrysophyceae	
<i>Mallomonas epithallatia</i>	Droop, unpublished
Bacillariophyceae	
* <i>Coscinoscira polychorda</i>	Droop, unpublished
* <i>Thalassiosira gravida</i>	Provasoli, unpublished
* <i>Stephanopyxis turris</i>	Provasoli, unpublished

* Pelagic species.

vitamin proper, B₁₂ III, and two artificial analogues (mammalian or *Ochromonas* pattern); one responded to all factors with the exception of the nucleotide-free factor B (*Lactobacillus leichmannii* or *Euglena* pattern); and three responded to all, including factor B (*Escherichia coli* pattern). It is possibly significant that the two diatoms are in this group. Two of the dinoflagellates were exceptional in being able to differentiate between pseudo-B₁₂ (containing adenine) and factor A (containing 2-methyladenine), responding to the latter and not the former. It is early to generalize; nevertheless, judging by these first results it is likely that the analogues of vitamin B₁₂ will have an importance equal to that of the vitamin.

Since most of the marine algae whose nutritional requirements are known are estuarine, littoral or supralittoral and not pelagic, it is impossible yet to assess the role of vitamin B₁₂ in the ecology of phytoplankton in general. A requirement for vitamin B₁₂ confined to neritic species and distinguishing them from their oceanic relatives might be a major factor in regional distribution. On the other hand, a seasonal control might be exerted on oceanic forms which require vitamin B₁₂ in any region where the concentration decreases

Table 4. *Specificity towards vitamin B₁₂-like factors*

Organism	Factor							
	B ₁₂ (5, 6-dimethylbenzimidazole)	5, 6-dichlorobenzimidazole analogue	Benzimidazole analogue	B ₁₂ III (Factor I)	Factor A (2-methyladenine)	Factor H (2-methylhypoxanthine)	Pseudo-B ₁₂ (adenine)	Factor B (No nucleotide)
<i>Prymnesium parvum</i>	+	+	+	+	0	0	0	0
<i>Microglena arenicola</i>	+	+	+	+	0	0	0	0
<i>Syracosphaera elongata</i>	+	+	+	+	0	0	0	0
<i>Isochrysis galbana</i>	+	+	+	+	0	0	0	0
<i>Hemiselmis virescens</i>	+	+	+	+	0	0	0	0
<i>Gyrodinium californicum</i>	+	+	+	+	0	0	0	0
<i>Gyrodinium</i> sp.	+	+	+	+	0	0	0	0
<i>Amphidinium klebsii</i>	+	+	+	+	+	+	0	0
<i>A. rhyncocephalum</i>	+	+	+	+	+	+	0	0
<i>Monochrysis lutheri</i>	+	+	+	+	+	+	+	0
<i>Amphora perpusilla</i>	+	+	+	+	+	+	+	+
<i>Skeletonema costatum</i>	+			+	+		+	+
<i>Phormidium persicinum</i>	+	+	+	+	+	+	+	+

+ = activity 25 % or more; 0 = activity less than 1 %; as compared with vitamin B₁₂.

sufficiently, a contingency more probable in the oceans than in coastal waters. Little has been done up to now on the seasonal and spatial distribution of vitamin B₁₂. The first assays of the content of coastal waters (Lewin, 1954; Droop, 1954, 1955 c) suggested figures of 5–10 mμg./l., which were high enough to support the heaviest known plankton crops; it appeared likely that vitamin B₁₂ would not be an important ecological factor after all. But more recently samples of water from the north North Sea and Norwegian Deepes taken over a period of 10 months have been assayed by Cowey (1956) who recorded values as little as 0.1 mμg./l.; these figures are low enough to hold promise of significant spatial and temporal differentials.

Marine vitamin B₁₂ is likely to be mainly of bacterial origin (Hall *et al.* 1950; Lochhead & Thexton, 1951; Ericson & Lewis, 1953), though reports of its production by *Chlorella* (Robbins, Hervey & Stebbins, 1951; Hashimoto, 1954; Brown, Cuthbertson & Fogg, 1956) indicate that the non-auxotrophic members of the phytoplankton and even benthic algae should not be entirely dismissed. Since marine bacteria have been found mainly associated with par-

ticulate matter (ZoBell, 1946) it is to be expected that coastal and estuarine areas and regions of upwelling will have the heaviest turnover.

To turn now to the sulphur requirements of diatoms; Harvey (1939) found that any of a number of compounds containing organic sulphur (cystine, glutathione, biotin, thiamine) were required for vigorous growth of *Ditylum brightwellii* in artificial sea water. Matudaira (1942) added inorganic sulphide to this list for *Skeletonema costatum*, and Harvey (1955) thiourea. All these experiments are to some extent open to criticism as they were carried out with bacterized cultures. Nevertheless, they were confirmed in principle with axenic cultures of the freshwater *Navicula pelliculosa* by J. C. Lewin (1954), who found that the washed diatoms were unable to assimilate silicon unless a compound containing divalent sulphur was added to the medium. Furthermore, sulphate was unable to restore their ability to assimilate but sulphate plus a reducing agent such as ascorbic acid did so.

A bacteria-free strain of *Skeletonema costatum* has now been maintained at Millport for nearly two years (Droop, 1955*b*). The defined medium contained thiamine, but on two occasions the diatom was passed through five transfers in the medium with thiamine omitted, without statistically-significant diminution in growth. It seems that there is no absolute requirement for thiamine or other organic sulphur compound. J. C. Lewin's (1954) experiment with ascorbic acid and sulphate suggests that the question of divalent sulphur might be tied up with redox phenomena. Some recent experiments at Millport support this view, as they show that the need for divalent sulphur is largely overcome when certain conditions of aeration are observed. For instance, vigorously aerated cultures of *S. costatum* require 3 mg. $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}/\text{l.}$, while still (non-aerated) cultures will not tolerate more than 1.0 mg./l. unless the medium has been allowed to stand some days before being used, and they grow as well without any added sulphide if the medium has not been allowed to stand too long. One finds the effect of sulphide is greatest when the inoculum has been taken from stationary phase cultures, and least when from logarithmic phase cultures. Harvey (1939) remarked the importance of the state of the inoculum. It seems that the sensitive period is the lag phase. The interdependence of sulphur requirements and oxygen tension may be reflecting the lability of the thiol radical in redox systems (the radical itself constituting the requirement) or may, on the other hand, mean that the function of these thiols is merely to maintain a critical redox potential at the cell surface.

In conclusion, I quote Provasoli & Pintner (1953); their statement 'it is a reasonable assumption that if an organism requires a growth factor *in vitro* then this metabolite or its physiological equivalent should be found in the environment' is an article of faith which has led to some useful results with vitamin B_{12} and will continue to do so with other compounds involving strictly trophic requirements. But compounds whose activity is physical rather than trophic (such as, possibly, the thiols) present a rather different problem, since their 'physiological equivalents' are likely to be numerous and their effects ill-defined and complex, and in some cases, no doubt, only of consequence in artificial cultures. Nevertheless, I believe these compounds

will prove as relevant to what Lucas (1949) has termed 'non-predatory' relationships among phytoplankton as any which are more simply trophically active.

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Relationships between Metabolism and Growth in Plankton Algae

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As an organism grows the pattern of its metabolism changes and, except under special conditions of culture, even the average chemical composition or activity of a population does not remain constant with time. Knowledge of the relationships between growth and metabolism in plankton algae is clearly worth having since the nature and intensity of the metabolic activities of these organisms in nature cannot generally be determined directly, but must be inferred from determinations of population densities and the chemical and physical properties of the waters in which they have developed.

Few truly planktonic algae have been isolated in pure culture and laboratory investigations on these have scarcely begun. For the present, therefore, we are forced to use information derived largely from studies with non-planktonic algae in interpreting the behaviour of plankton. It cannot be assumed, of course, that planktonic algae always behave in a similar manner to the sewage alga *Chlorella* for example, but the now quite substantial body of information about the metabolism of this and a few other algae is beginning to give a picture which is probably of general application. It is my purpose in this paper to sketch the outlines of this picture and to discuss some of its applications to plankton.

When an alga is inoculated into a limited amount of culture medium and exposed to suitable conditions of light, temperature and aeration, it shows more or less well-defined phases of growth following each other in the familiar pattern, i.e. there is a lag phase, an exponential phase, a phase of declining relative growth rate and a stationary phase. Few studies have been made on the lag phase in algae but it has been found in some species that the cell protein and nucleic acid contents increase and we may surmise that this phase is one of reconstitution, in which enzyme and substrate concentrations are built up to the degrees necessary for multiplication. In the exponential phase the organisms have a high capacity for photosynthesis and the products are used mainly for the synthesis of protein. In a continuous culture, in which the population density is kept constant by the regulated addition of fresh medium, this phase of growth can be maintained indefinitely with the chemical composition and physiological attributes of the organisms remaining constant. In a culture of limited volume, however, the exponential phase ends after a time because of depletion of nutrients, accumulation of toxic by-products of metabolism, or simply because light becomes limiting as the culture becomes more dense.

By the end of the exponential phase the capacity of the organisms for

photosynthesis has declined considerably and its products become increasingly diverted along pathways other than that of protein synthesis to form 'reserves' of fat or carbohydrate. These changes are not abrupt but gradual, beginning well before exponential increase in number of organisms ceases. Thus the decline in photosynthetic capacity begins a day or two before that of relative increase in numbers of organisms. The plotting on a logarithmic grid of the total amounts of various fractions in cultures as a function of dry weight generally yields straight lines, showing that the differential rates of increase of the different components remain constant over almost the whole period of culture. It seems that constant relative increase in number of organisms in such cultures does not represent a steady state condition but that it may be maintained while considerable changes of cell composition in terms of major fractions such as protein, fat and carbohydrate are taking place. The direction of these changes depends on culture conditions; we have a great deal to learn about this still but it seems generally true that when nitrogen is limiting the products of photosynthesis are used by algae for fat synthesis, whereas when phosphate is in short supply carbohydrate is accumulated rather than fat.

There is increasing evidence that extracellular products of algae are often important quantitatively and also because of their biological effects. All algae which have been examined from this point of view have been found to liberate peptides into the medium during the course of growth (Fogg & Westlake, 1955). The relative amount of these substances is increased by deficiencies of certain elements and can account for as much as 50 % of the total nitrogen assimilated, but their liberation seems to be invariably linked with growth and to be independent of the volume of the medium. Organic acids are also prominent among the extracellular products of at least some species. Glycollic acid, for example, has been found to be liberated in substantial amounts from photosynthesizing *Chlorella* (Tolbert, 1955) and this and other organic acids have been found to accumulate in amounts corresponding to 10–45 % of the total organic material formed in cultures of various *Chlamydomonas* spp. (Allen, 1956). It is to be expected that, with metabolites of low molecular weight like organic acids, there is an equilibrium between the intra- and extracellular concentrations so that the relative amount liberated from within the organisms should be dependent on the volume of the medium. Such a relationship, however, does not appear to have been demonstrated experimentally. Evidence is accumulating to show that many algae produce antibiotic extracellular substances (Lefèvre, Jakob & Nisbet, 1952; Steemann Nielsen, 1955), but the chemical nature of these and the physiological relations in their production have yet to be established.

Some of the applications of these findings to plankton may now be considered. A phase of protoplasmic growth without cell division, resembling the lag phase in cultures, is probably necessary before populations of plankton algae begin to increase but, on the face of it, appears difficult to study directly. Possibly such a lag phase is responsible for the phenomenon of the 'spring outburst'. It is well known that many micro-organisms cannot begin exponential growth until particular concentrations of certain metabolites have been

established in the external medium. Were a similar state of affairs to exist for phytoplankton increase of numbers would not necessarily occur under favourable conditions of light, temperature and nutrient supply, but would have to await the establishment of the requisite concentration of metabolites in the water immediately surrounding the organisms. This explanation is consistent with the observed correlation between the spring outburst and decreasing turbulence.

Something resembling exponential growth occurs in natural phytoplankton populations, but whether or not the same sort of variation in metabolic pattern and activity of the organisms occurs as in laboratory cultures remains to be demonstrated. A phase of intense photosynthetic activity similar to that observed at the beginning of the exponential phase in culture may be difficult to detect on account of its transient nature and the low population density. Recorded maximum rates of photosynthesis of natural phytoplankton populations evidently all represent later stages of growth since they are nearly all of about the same magnitude and about one-third of those recorded for actively growing laboratory cultures of *Chlorella* when expressed on the basis of oxygen evolution per unit volume of cell material or per unit weight of chlorophyll (Verduin, 1952; Ryther, 1956). In the later stages of exponential growth and in the phase of declining relative growth, such as are most usually encountered in phytoplankton populations, the products of photosynthesis may be expected to be used mainly in synthesis of 'reserve' products. In phytoplankton these are usually fats and their synthesis would give a photosynthetic quotient of the order of 1.4. Thus if a value of unity be assumed for the photosynthetic quotient, as is usual in studies on plankton productivity, the amount of carbon fixed as calculated from observed oxygen evolution may be considerably overestimated. Ryther (1956) has considered this problem and has concluded that a value of 1.25 for the photosynthetic quotient would be more realistic.

There are, of course, important differences between the growth of natural phytoplankton populations and that of algae in laboratory cultures of limited volume. Interaction with other organisms may result in considerable modification of growth and metabolism. Zooplankton decreases phytoplankton numbers by grazing but also, together with bacteria, continuously liberate plant nutrients so that phytoplankton multiplication takes place even though there are no detectable amounts of these nutrients in the water. On the other hand, staling products which would accumulate in pure cultures may be removed or otherwise inactivated by associated organisms. Thus a natural phytoplankton population which is stationary as far as numbers/unit volume is concerned is not likely to be physiologically equivalent to a stationary population in laboratory culture. Moreover, a natural population is not limited to a definite volume of water but can expand into the surrounding water. In that it may have a continuous supply of nutrients whilst its staling products are continually removed or diluted, a natural phytoplankton population perhaps resembles in its physiological condition that in continuous culture more closely than that in a limited volume of medium.

Extracellular products of plankton are of interest in several ways, but mention can only be made here of their possible significance in relation to productivity studies. If there is indeed a tendency towards equilibrium between intra- and extracellular concentrations of certain metabolites then it might be that the loss of such substances from the organisms at low plankton densities might be relatively great so that methods such as the radiocarbon method of Steemann Nielsen, which take account only of carbon fixation in particulate matter, may seriously underestimate the total productivity. This might account for the discrepancy between the results given by the Steemann Nielsen method on the one hand and the Gaarder and Gran method on the other, for oligotrophic waters. Preliminary studies with radiocarbon as a tracer have shown that the amount of photosynthetically-fixed carbon in filtrates of a eutrophic lake water is only about 0.5–1 % of that in the plankton retained on the membrane filter and that this proportion does not increase as the plankton concentration is decreased. However, we know too little about the physiology of the extracellular products of algae to be sure that a similar relation holds for different physiological states and for all types of plankton.

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CORRIGENDUM

In STRANGE, R. E. and DARK, F. A. (1957). *J. gen. Microbiol.* **16**, 236-249.

On p. 245, Table 7:

for: 'g./100 g. cell-wall'

read: 'g./100 g. cell-wall peptide'

KLEMPERER, R. M. M. (1957). *J. gen. Microbiol.* **16**, 299-304

The Normal Distribution of the Resistance of Coliphage T3 to Inactivation by Bacterial Receptors and its Application to their Assay

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SUMMARY: A study of the inactivation of coliphage T3 by its bacterial receptors indicates that the resistance of the phage is variable and conforms to a log normal distribution. Receptor activity can be conveniently assayed using probits to obtain a linear relation between phage survival and receptor concentration. This method is sufficiently sensitive to distinguish between the reactivity of receptors prepared from different bacterial hosts.

Fildes & Kay (1955), studying adsorption of phage 1 by strains of *Salmonella typhi*, found that the plot of the log % phage unadsorbed against time was linear. They obtained the same result for the inhibition of phage 1 by a relatively pure receptor preparation. The time required for the inhibition of 50 % phage was inversely proportional to the receptor concentration, and could be used to estimate receptor concentration. In the present paper it is shown that these relations do not hold for the reaction of coliphage T3 with its receptors. However, it has been found possible to determine receptor concentrations by assuming that the resistance of coliphage T3 particles to inactivation is not uniform but is normally distributed.

METHODS

Organisms. *Escherichia coli* B, obtained originally from Dr T. F. Anderson, was kept on Dorset egg medium at room temperature and subcultured every 3 months. Cultures for immediate use were grown on tryptic meat agar from stock cultures.

Salmonella typhi O901R, originally selected from O901S (Fildes & Kay, 1955), was cultivated in the same way.

Coliphage T3 in high titre was kindly provided by Dr D. Kay (Kay, 1955) and stored at 4°. Tenfold dilutions in buffer (see below) were stored at 4° to provide inocula for daily use. Titres remained constant for many months.

Estimation of phage. Phage particles were counted by the two-layer method of Hershey, Kalmanson & Bronfenbrenner (1943), 1 ml. phage + bacteria mixture being added to 2 ml. peptone agar to make the surface layer.

Preparation of bacterial extracts. For the preparation of extracts of *Escherichia coli* B, the organism was grown in a Difco Bacto-Casamino acid medium (pH 7.6), supplemented with lactate, glycerophosphate, tryptophan, magnesium, manganese, iron and yeast extract, and aerated with air + 5 % CO₂ in an apparatus as described by van Heyningen & Gladstone (1953). The

organisms were centrifuged off and washed once with water. They were then resuspended in a small volume of water and precipitated with 6 vol. acetone. This was repeated twice more. The precipitate was dried in a desiccator (yield: 4–5 g./l. culture). Dried organisms were extracted 3 to 6 times at 56° for 1 hr. with successive volumes of water, using 10 ml. volumes/g. organisms. The pooled extracts were precipitated with acetic acid at pH 4, the precipitate redissolved in water and the pH value adjusted to 7.6 with NaOH. The final volume of extract was one-tenth the original volume. The acetic acid precipitation increased the activity per unit volume up to 25 times, in spite of the loss of some activity in the supernatant fluid. O901R extracts were prepared in the same way.

An O901R extract which had been further purified by sodium chloride precipitation was provided by Dr D. Kay. Unlike the material previously described by him (Kay, 1955), it had a high anti-phage T3 activity, probably because it was the result of a large number of extractions of the acetone-dried organisms.

Buffer. This contained: phosphate (pH 7.6) M/30 (autoclaved); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ M/4000 (autoclaved); albumin (human) 1/2500 (filtered).

Stock concentrated solutions were sterilized separately and mixed to give the indicated concentrations.

Estimation of receptor activity in bacterial extracts. A sample (2 ml.) of phage (5 to 10×10^3 particles/ml.) in buffer was incubated for 30 min. at 37°. During this time the phage count rose owing to disaggregation of the particles. An equal volume of bacterial extract in buffer was then added, the extract having previously been diluted to give various concentrations of receptors. Samples (0.5 ml.) were withdrawn immediately and after the required time of incubation, and pipetted into 4.5 ml. 1% (w/v) peptone water to stop the reaction. One vol. of the diluted material was added to 2 vol. *Escherichia coli* B culture in peptone water, and the number of phage particles counted (in duplicate or triplicate).

Expression of results. Receptor concentration is expressed throughout as the concentration of bacterial extract used as the source of receptor. In Figs. 2–6, receptor concentrations are expressed as \log_2 reciprocal of extract concentration, i.e. $-\log_2$ extract concentration. All straight lines are calculated as regressions of ordinate upon abscissa. Correlation coefficients were high in all instances ($P = < 0.001$ in Figs. 3, 4 and 6, and < 0.01 in Fig. 5).

RESULTS

Reaction of coliphage T3 with bacterial extracts

When coliphage T3 was incubated with receptor preparations the log percentage phage survival, i.e. unadsorbed phage, fell rapidly during the first minutes of incubation, but later only diminished slowly (Fig. 1). Thus the rate of phage inactivation decreased rapidly with the fall in the concentration of free phage. The plot of percentage phage survival after 30 min. incubation against the log receptor concentration is a sigmoid curve (Fig. 2). This sigmoid curve is

of the same type as an integrated frequency curve for a normal distribution, since probit percentage phage survival plotted against log receptor concentration gives a straight line (Figs. 3, 4) (Bliss, 1935).

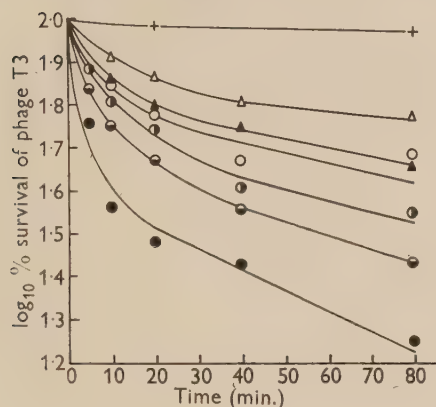


Fig. 1

Fig. 1. Inactivation of phage T3 by coli B receptors. Bacterial extract added: $++$, 0; $\triangle-\triangle$, 1/320; $\blacktriangle-\blacktriangle$, 1/160; $\circ-\circ$, 1/120; $\bullet-\bullet$, 1/80; $\ominus-\ominus$, 1/40; $\bullet-\bullet$, 1/20.

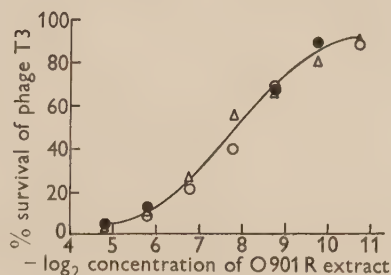


Fig. 2

Fig. 2. Inactivation of phage T3 by O901 R receptors after 30 min. incubation. The extract used was acid and salt precipitated. The curve is based on results from triplicate experiments represented by \bullet , \circ and \triangle .

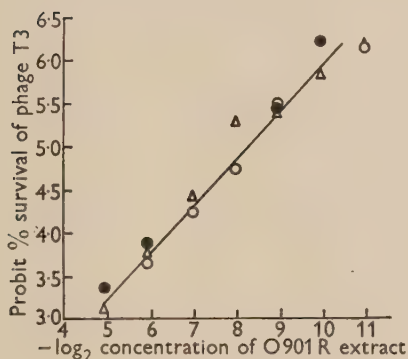


Fig. 3

Fig. 3. Inactivation of phage T3 by O901 R receptors after 30 min. incubation. \bullet , \circ and \triangle refer to the same experimental points as in Fig. 2. Regression coeff: 0.53; standard error ± 0.031 . When probit % phage survival is 5.0, $-\log_2$ extract concn. (with 95 % confidence limits) is 8.18 ± 0.11 , i.e. titre of extract is 1/290 (95 % confidence limits: 1/270-1/315).

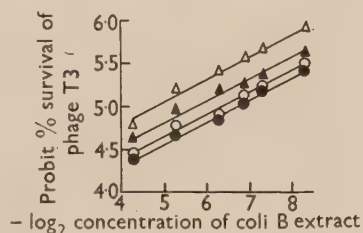


Fig. 4

Fig. 4. Inactivation of phage T3 by coli B receptors. These points are calculated from the curves shown in Fig. 1.

	Incubation (min.)	Regression coeff.	Standard error
$\triangle-\triangle$	10	0.27	± 0.016
$\blacktriangle-\blacktriangle$	20	0.24	± 0.012
$\circ-\circ$	30	0.25	± 0.016
$\bullet-\bullet$	40	0.25	± 0.010

Estimation of receptors

The use of probits provides a convenient method for the assay of receptor activity. The amount of receptor required for 50 % inactivation of phage can be estimated accurately from the plot of probit percentage phage survival against log receptor concentration. Thus in Fig. 5, the increased concentration of extract required for 50 % inactivation of phage in the presence of antiserum indicates approximately one-third reduction in receptor activity.

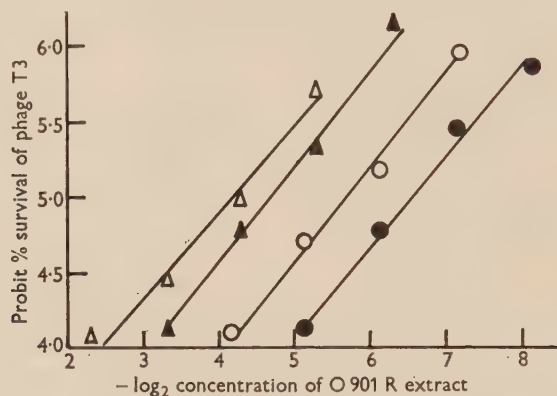


Fig. 5. Inactivation of phage T3 by O901 R receptors after 30 min. incubation. Bacterial extracts, at a concentration *c.* 20 times that required to inactivate 50 % phage, were incubated with equal volumes of an anti-O901 R serum diluted 1/8 in buffer, or with equal volumes of buffer only for 30 min. at 37°. They were then diluted and assayed for receptor activity as usual. The serum was prepared by repeated intravenous injections of heat-killed bacteria into a rabbit.

O901 R extract	Anti-serum	Regression coeff.	Standard error	$-\log_2$ extract concn.*	Therefore titre of extract	95 % confidence limits
Acid precipitated						
$\triangle-\triangle$	+	0.56	± 0.057	4.21 ± 0.27	1/18.5	1/15-1/22
$\blacktriangle-\blacktriangle$	0	0.65	± 0.035	4.69 ± 0.16	1/26	1/23-1/29
Same preparation as in Fig. 3, but stored 7 months at 4°						
$\circ-\circ$	+	0.59	± 0.052	5.73 ± 0.24	1/53	1/45-1/63
$\bullet-\bullet$	0	0.59	± 0.050	6.58 ± 0.21	1/96	1/83-1/110

* When probit % phage survival = 5.0 (with 95 % confidence limits).

Reactivity of receptors

The slope of the line relating probit percentage phage survival to log receptor concentration remained constant using different preparations of a receptor. Thus Fig. 5 shows that the slope for O901 R extract was unaffected when the activity was reduced one-third by treatment with antiserum. Comparison of Fig. 5 with Fig. 3 shows that the slope was also unaltered by further purification of the receptor, or when the receptor was approximately two-thirds inactivated by prolonged storage. Similar experiments showed that the slope for different coli B receptor preparations was also constant.

However, receptors from the two bacterial species tested were different. This is shown by the observation that the slope of probit percentage phage

survival against log concentration of O901 R receptor (Fig. 3) is always twice as great as that for coli B receptors (Fig. 4). (The regression coefficients differ significantly: $P=0.02-0.01$; Student's t test.)

DISCUSSION

The sigmoid relation between the survival of coliphage T3 and the log receptor concentration suggests that the coliphage T3 particles are heterogeneous in their resistance to inactivation. As the plot of the probit percentage phage survival against log receptor concentration is linear, the resistance of the phage particles to inactivation may be assumed to be normally distributed in relation to the log receptor concentration. In this respect, the resistance of coliphage T3 to inactivation resembles that of bacteria, animals and plants to toxic agents (Gaddum, 1933; Withell, 1942). It would be expected from Withell's work that the resistance of phage particles to inactivation would also be normally distributed with respect to log time for any concentration of receptor. The results given here are in accordance with this when plotted appropriately, but do not extend over a long enough period of time to be conclusive.

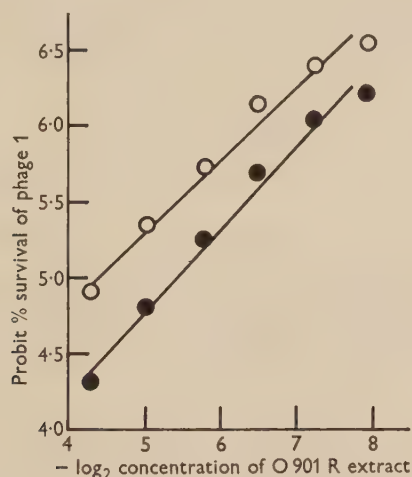


Fig. 6. Inactivation of phage 1 by O901 R receptors. These points are calculated from data supplied by Dr D. Kay. The extract used was acid and salt precipitated.

	Incubation (min.)	Regression coeff.	Standard error
○—○	10	0.49	± 0.034
●—●	20	0.56	± 0.034

The plot of log percentage survival against time was not linear for coliphage T3, unlike the linear relationship found by Fildes & Kay (1955) for phage 1. A linear relationship is of the same type as that of a first-order reaction in which the reactants are uniform. However, the behaviour of phage 1 may also be interpreted in terms of a normal distribution since the relationship between

probit percentage survival and log receptor concentration is linear (Fig. 6: data kindly supplied by Dr Kay). This is in agreement with the findings of Withell (1942) that any observed form of time-survival curve can be represented approximately as a normal distribution when time or concentration of the toxic agent is plotted logarithmically. It suggests that phage 1 may also be heterogeneous like coliphage T3. The heterogeneity of phages in other respects is well known. For example, Burnet, Keogh & Lush (1937) found in several cases that phages varied in their resistance to inactivation by antibody, some showing typical log normal distributions.

Burnet *et al.* (1937) showed that the receptor for *Shigella flexneri* phage was heterogeneous in its ability to combine with phage, and in general crude extracts were more avid than purer fractions. No evidence was obtained in the experiments described here of any difference in the reactivity of different receptor preparations. However, it was of interest that the slopes of inactivation of coliphage T3 by receptors prepared from two bacterial hosts were different. It is possible that the steeper slope obtained with O901R receptor is due to greater avidity.

This work was carried out in collaboration with the British Empire Cancer Campaign Virus Research Unit, and my thanks are due to Sir Paul Fildes, F.R.S., the Director, for his interest in it. I also wish to thank Dr G. P. Gladstone and Dr D. Kay for much useful advice. The work was done during the tenure of a Phillip Walker Studentship.

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The Oxidases of *Gelasinospora tetrasperma* in relation to the Endogenous Respiration

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SUMMARY: The active growth of *Gelasinospora tetrasperma* in a chemically defined liquid medium was completed in 5 days. Cytochrome was detected at all ages (spectroscopically and by manometric assay) and a cytochrome-cytochrome oxidase system was mainly responsible for O₂ uptake. Phenol oxidase was not detected until the cultures had been growing 48 hr., and even then the proportion of the O₂ uptake of the mycelium due to this enzyme was small, since light reversed carbon monoxide inhibition of the O₂ uptake almost completely.

Whereas the importance of cytochrome *c* oxidase as a terminal oxidase of plant respiration is now firmly established, the role, if any, of phenol oxidases in plant respiration is uncertain (James, 1953*a*). In the Basidiomycete *Poly-stictus versicolor*, which produced both cytochrome *c* oxidase and a phenol oxidase, laccase, only the former enzyme was involved in oxygen uptake (Boulter & Burges, 1955). The present work was undertaken to assess the relative importance of cytochrome *c* oxidase and the phenol oxidase, tyrosinase, in the endogenous respiration of *Gelasinospora tetrasperma*, an Ascomycete, which produces both these enzymes.

METHODS

Culture medium. *Gelasinospora tetrasperma* Dowding, obtained from Dr Dowding, was grown in a liquid medium of the following composition: D(+) glucose, 10 g.; asparagin, 5 g.; KH₂PO₄, 0.5 g.; Mg₂SO₄·7H₂O, 0.5 g.; NaCl, 0.25 g.; biotin, 50 µg.; thiamine, 100 µg.; distilled water to 1 l. (glucose autoclaved separately; thiamine sterilized by filtration).

Inoculum. Mycelial suspensions from 48 hr. cultures, grown in 40 ml. medium at 25°, were used for inoculations. Mycelium from two flasks was washed and transferred with 40 ml. water to the metal cup of an Atomix blender (M.S.E., London) and blended at full speed for 10 sec. This agitation gave a fine mycelial suspension which was washed twice by centrifugation and resuspended in water; 1 ml. of suspension was used to inoculate each 250 ml. flask containing 40 ml. medium.

Culture conditions. Cultures were incubated at 25° on a reciprocal shaker (stroke 4 in., 75 oscillations/min.).

Chemicals. Substrates and inhibitors were obtained from the British Drug Houses Ltd., and were of analytical grade when available. Acid and alkaline compounds were neutralized; unstable substrates were recrystallized and

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fresh solutions of them made up just before use. O_2 and N_2 (O_2 free) were obtained in cylinders from the British Oxygen Co. Carbon monoxide was made by the action of concentrated sulphuric acid on formic acid and oxygen was removed from the evolved gas and from nitrogen by passage through alkaline pyrogallol triacetate and then through an acid and a water wash. Gas mixtures were made in 10 l. bottles by displacement of water.

Spectroscopic examinations were made with a Hilger micro-spectroscope, using a 500 W. metal filament lamp and allowing light to pass through thick suspensions of the mycelium to which sodium dithionite had been added.

Preparation of extracts. All operations were carried out between 0° and 4° . Fungal cultures of various ages were harvested and washed on a Buchner funnel with 150 vol. (w/v) of distilled water. Mycelium to be extracted for the examination of cytochrome *c* oxidase activity was ground with phosphate buffer in a mortar (1 part moist mycelium to $\frac{1}{3}$ part (w/w) quartz and 5 parts (w/v) 0.05 M-phosphate buffer (pH 7.5) containing 0.001 M-ethylenediamine-tetraacetic acid (Versene)). The suspension was centrifuged at 1000 *g* for 10 min. to remove cell debris and quartz and the supernatant fluid (H) then centrifuged at 5000 *g* for 20 min. This supernatant fluid (SN) was discarded and the residue (P) resuspended in 0.05 M-phosphate buffer (pH 7.5) containing Versene and centrifuged again for 20 min. at 5000 *g* to give a pellet which was suspended in one-third the original volume of 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene (P_1).

Extracts to be tested for Dopa oxidase activity were prepared by washing the mycelium as before and grinding it in a mortar with a minimum of quartz and buffer (1 part moist mycelium to 9 parts (w/v) 0.05 M-phosphate buffer to give pH 7.0). The cloudy suspension was centrifuged for 10 min. at 1000 *g* and the residue of quartz, cell debris and unbroken cells re-extracted with half the previous volume of buffer, allowed to stand 10 min. and centrifuged again at the same speed for 10 min. The two supernatant fluids were combined and dialysed against 0.01 M-phosphate buffer (pH 7.0) overnight at 2° . The extracts were adjusted to pH 7.0 before testing.

To determine the intracellular localization of Dopa oxidase activity, cell fractions were prepared from 4-day mycelium by grinding with quartz and phosphate buffer. The ground mycelium was centrifuged gently to give a cell-wall fraction (W_0) and a supernatant (SN_0). (SN_0) was centrifuged at 5000 *g* for 20 min. to give a supernatant (SN_1) and a particle fraction (P). The fraction (W_0) was re-extracted and allowed to stand 10 min. and the suspension then recentrifuged gently to give a cell-wall fraction (W_2) and a supernatant (SN_2). Further re-extractions of (W_0) were designated (W_3), (W_4) and (SN_3), (SN_4). The fractions were dialysed against 0.01 M-phosphate buffer (pH 7.0) overnight at 2° and adjusted to pH 7.0 before testing.

Measurement of enzyme activity of extracts. Cytochrome *c* oxidase activity was measured as the rate of oxygen uptake in Warburg manometers at 25° . The flasks contained the enzyme extract; cytochrome *c* (Sigma Chemical Co., U.S.A.); 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 0.1 M-hydroquinone in the side arm and 0.15 ml. 15 % (w/v) KOH on filter-paper

in the centre well. After tipping the side arm, readings were taken at 5 min. intervals for 20 min. and the activity/hr. calculated from the initial rate of O_2 uptake. The O_2 uptake due to autoxidation of the hydroquinone was subtracted and was estimated by extrapolating to zero enzyme concentration at constant cytochrome *c* concentration (Umbreit, Burris & Stauffer, 1949). Autoxidation of hydroquinone occurred even when Versene was present in the assay and grinding media. In order to find the maximum oxidase activity at a constant enzyme concentration, the O_2 uptake with a constant enzyme concentration was measured with different concentrations of cytochrome *c*. The reciprocal of the O_2 uptake values was then plotted against the reciprocal of the cytochrome *c* concentration and the maximum O_2 uptake at this enzyme concentration was calculated by extrapolating to infinite cytochrome *c* concentration (Lineweaver & Burk, 1934). This maximum cytochrome *c* oxidase activity was expressed as $\mu l.$ O_2 uptake/hr./mg. N (Q_{O_2} max. (N)).

Nitrogen determinations of the enzyme preparations were made by precipitating nitrogen compounds with 10 % (w/v) trichloroacetic acid, digesting the washed precipitate with concentrated H_2SO_4 + one drop of H_2O_2 and determining the nitrogen by Nessler's method.

Dopa oxidase activity was measured at a wavelength of 480 $m\mu$. at room temperature, using a Unicam S.P. 500 spectrophotometer. Glass cells of light path 1 cm. were used containing in a total volume of 3.0 ml.: enzyme; DL-3:4-dihydroxyphenylalanine (Dopa) and 0.01 M-phosphate buffer (pH 7.0). The enzyme was added to the assay system and readings at 10 sec. intervals were commenced 15 sec. after the addition. During the period of the first few readings, the oxidase activity remained approximately constant and the activity/hr. was calculated on this initial rate.

The Dopa oxidase activities of highly turbid suspensions were measured in an EEL colorimeter with a blue filter. Cells contained in a total of 6.0 ml.: enzyme extract; 0.005 M-Dopa and 0.05 M-phosphate buffer (pH 7.0). The substrate was temperature equilibrated (25°) separately, added to the rest of the system and readings taken at 2 min. intervals. The cells were returned to the water bath in between readings and continuously shaken gently. Autoxidation of substrate and maximum activity were calculated as for cytochrome *c* oxidase. The activity was measured as the change in optical density/min./mg. N in the Unicam and as the change in EEL units/hr./mg. N in the EEL colorimeter. Manometric checks with larger volumes of enzyme were made to ensure that reducing substances were not grossly distorting the assays.

Respiration measurements on intact mycelium. The respiration was measured at 25°, in the presence and absence of inhibitors, by determining the oxygen uptake in Warburg manometers by the direct method (Dixon, 1943). O_2 uptake was referred to mg. dry wt. mycelium, obtained by washing the mycelium from the Warburg flasks on to weighed sintered-glass crucibles, and drying these overnight at 98°.

Application of inhibitors. Inhibitors of the oxidases were used at pH values and concentrations that ensured maximum effectiveness and selectivity (James, 1953b). Inhibition of cytochrome *c* oxidase, tyrosinase and the O_2

uptake of the intact mycelium by various gas mixtures, was measured in Warburg manometers; gas mixtures were introduced into the flasks by an evacuation technique (Umbreit *et al.* 1949). When required, flasks were darkened during temperature equilibration and during dark periods by a dark cloth thrown over the top of the bath. The light source used in the carbon monoxide experiments was a 250 W. mercury vapour lamp and when in use it was necessary to cool the system with a powerful fan. The inhibition was calculated as the percentage decrease of the O_2 uptake in a particular gas mixture, as compared with the O_2 uptake of control samples in air.

The effects of phenylthiourea and KCN on cytochrome *c* oxidase activity and on the O_2 uptake of the mycelium were also measured in Warburg manometers. After a steady rate of O_2 uptake had been established, the inhibitors were added to the contents of the Warburg flasks, phenylthiourea from the side-arm and KCN (at 25°) directly from the outside. In the flasks to which KCN had been added, 3 min. were allowed for temperature equilibration before re-reading the manometers. Inhibitions were calculated as the percentage decrease in O_2 uptake of the sample on addition of the inhibitor. KCN inhibition was calculated from the initial rate of O_2 uptake on adding the inhibitor, as the inhibition quickly diminished due to KCN distilling into the KOH of the centre well.

The effects of KCN, phenylthiourea and Na diethyldithiocarbamate on Dopa oxidase activity were measured spectrophotometrically and calculated as the percentage decrease in activity of samples in the presence of the inhibitors, compared with control samples in their absence.

RESULTS

Factors affecting cytochrome c oxidase activity

Preliminary experiments, performed to ascertain the best conditions for obtaining the maximum extraction and preservation of oxidase activity and the optimum conditions for assay, are not recorded in detail, but they accorded in general with the experience of other workers using cytochrome *c* oxidase prepared from different sources (Hartree, 1955).

Grinding the mycelium with quartz by hand in a mortar gave higher oxidase activities than homogenization with a glass homogenizer, the use of the Atomix blender or hand grinding in a mortar with other abrasives. Maximum activity was found when the temperature was kept as near 0° as possible and when the pH value did not drop below pH 7.0. When cell-free homogenates were kept at room temperature, inactivation of cytochrome *c* oxidase took place. This inactivation was less rapid at 2°; but for maximum activity it was necessary to remove the oxidase from the homogenate as soon as possible. This was done by centrifuging at 5000 *g* for 20 min. and then washing the particles which sedimented by resuspending them in the grinding media and re-centrifuging. The results (Table 1) showed that 77% of the oxidase activity was in the particle fraction. This value was only approximate however, as some inactivation took place in the homogenate as evidenced by the lower activity

of the homogenate when compared with the combined activities of the other two fractions.

Table 1. *Localization of cytochrome c oxidase activity within the cells*

Cell-free preparations from 36 hr. mycelium as described in text. Warburg manometer flasks contained in a total vol. of 2.0 ml.: 0.5 ml. extract; 10–50 μ M-cytochrome *c*; 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 0.1 M-hydroquinone in side arm and 0.15 ml. 15 % (w/v) KOH in centre well. Gas phase: air; temp. 25°. Recorded O₂ uptake as the calculated maximum corrected for autoxidation (see Methods).

Cell fraction	μ l. O ₂ uptake/hr.	μ l. O ₂ uptake/hr./mg. N
Cell-free homogenate (H)	11,000	1,160
Supernatant (SN)	2,750	550
Particles (P ₁)	9,250	2,000

The endogenous O₂ uptake of washed particle preparations, without added cytochrome *c* and hydroquinone, or with either alone, was negligible. Substrate concentrations greater than 0.08 M-hydroquinone did not give increased O₂ uptake at constant cytochrome *c* concentration; 0.1 M-hydroquinone was therefore used throughout. The concentration of enzyme preparations varied.

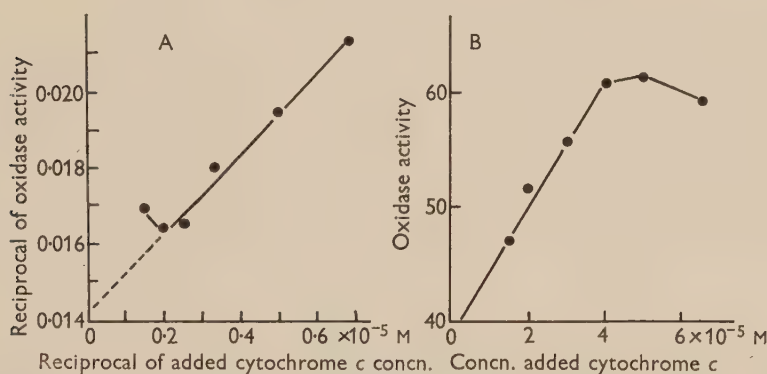


Fig. 1. The effect of cytochrome *c* concentration, on the activity at constant cytochrome oxidase concentration. Extract prepared from 48 hr. mycelium, as described in text, except the washed particles were resuspended in the original volume of buffer used for grinding. Warburg manometer flasks contained in a total vol. 2.5 ml.: 1.0 ml. extract; cytochrome *c* as indicated; 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 0.1 M-hydroquinone in side arm and 0.15 ml. 15 % (w/v) KOH in centre well. Gas phase: air; temp. 25°. Activity expressed as μ l. O₂ uptake/hr./ml. extract.

Normally, with constant enzyme concentration, enzyme activity increased with increasing cytochrome *c* concentration. However, with less concentrated enzyme preparations, maximum activity was found with cytochrome *c* concentrations of the order 50 μ M; higher cytochrome *c* concentrations gave lower activities (Fig. 1). In those instances, Q_{O_2} max. was calculated from the straight line part of the curve and the observed optimum Q_{O_2} with saturating cytochrome *c* concentration was not used (Lineweaver & Burk, 1934). The optimum pH value of the enzyme with 0.01 M-phosphate was pH 7.2.

Factors affecting Dopa oxidase activity

Unlike cytochrome *c* oxidase, Dopa oxidase activity was found mainly in the supernatant fraction (Table 2). The enzyme was therefore exposed continuously, from breaking the cells until completion of assay, to any inhibitors or denaturing compounds which might have been present in the crude homogenate.

Table 2. *Localization of Dopa oxidase activity within the cells*

Details of cell-free preparations in text. EEL colorimeter cells contained in total of 6.0 ml.: 1 ml. extract; 0.005 M-Dopa and 0.05 M-phosphate buffer (pH 7.0). Fraction W₄ was centrifuged down in assay cell.

Fraction	EEL units/hr./ fraction	EEL units/hr./ mg. N
Supernatant (SN ₀)	4080	350
Cell wall (W ₀) (by calculation)	1000	—
Supernatant (SN ₁)	4080	500
Particle (P)	0	—
Supernatant (SN ₂) (2nd extract)	620	460
Supernatant (SN ₃) (3rd extract)	200	210
Supernatant (SN ₄) (4th extract)	80	40
Cell wall (W ₄) (after 4th extract)	100	—

Although enzyme activity was found mainly in the supernatant fluid, some activity was only removed from the cell wall + quartz fraction by repeated re-extractions of this fraction with buffer. However, after a single re-extraction of the debris fraction, 81 % of the total activity was in the soluble fractions and with further re-extraction of the debris all but 2 % was found in the soluble fraction. No Dopa oxidase activity was associated with the particle fraction. During these experiments the enzyme, when stored in the cold, increased in activity; this increase depended on the pH value during extraction and storage.

Table 3. *The effect of pH of extraction and dialysis on the Dopa oxidase activity of extracts*

Extracts made from 3-day mycelium. Activity tested immediately and after dialysis overnight at 2° against 0.01 M-phosphate buffer. Extracts adjusted to pH 7.0 before assay. Spectrophotometer cells contained in a total of 3.0 ml.: 0.2 ml. enzyme extract; 0.005 M-Dopa and 0.05 M-phosphate buffer (pH 7.0). Extract preparation and assay details in text.

pH during extraction	pH of dialysis buffer	pH of prepara- tion after dialysis	μg. N/ ml.	Δ o.d./ min./ mg. N
7.0	Assayed at once	—	98	0.85
	7.0	7.0	90	4.7
	6.0	6.2	85	3.6
	5.0	5.4	60	0.98
	Overnight at 2°—no dialysis	—	95	4.0
6.0	Assayed at once	—	96	0.73
	6.0	6.1	78	3.1
	Overnight at 2°—no dialysis	—	90	2.5

Table 3 gives the Dopa oxidase activities of preparations extracted from the mycelium at various pH values at 2°. The greatest activity was found with preparations which had been extracted and dialysed at pH 7.0, although the initial activities of preparations extracted at pH 7.0 and 6.0 were not greatly different. The possibility that the increase in activity of dialysed preparations was due to the removal of a dialysable inhibitor was excluded since the only effect of undialysed preparations on the activity of dialysed ones was to cause a lag period, varying from several seconds to minutes, before oxidation of substrate took place. When the preparations were not dialysed, but left overnight at 2° at the pH value of extraction, an increase in activity occurred which was nearly as great as that in similar preparations which had been dialysed.

Table 4. *The effect of 'ageing' conditions on the Dopa oxidase activity of cell-free extracts*

Extracts made from 4-day mycelium and tested after 'ageing' as indicated. Spectrophotometer cells contained in a total of 3.0 ml.: 0.2 ml. extract; 0.005M-Dopa and 0.05M-phosphate buffer (pH 7.0). Extract preparation and assay details in text.

Conditions of 'ageing'	Δ o.d./min./ 100 μ g. N
Tested at once	0.09
After 1 hr. at 27°	0.16
After 3 hr. at 27°	0.18
After 8 hr. at 27°	0.32
After 12 hr. at 27°	0.32
After 1 hr. at 2°	0.13
After 8 hr. at 2°	0.35
After dialysis overnight at 2°, pH 7.0	0.39
After dialysis overnight at 2°, pH 7.0, then left for 3 days at 2°	0.39
After 8 hr. at 2° and 12 hr. at 27°	0.33

Maximum activity was found after 8 hr. at 2° and this activity was stable for at least several days in dialysed preparations stored at pH 7.0 and 2°. Dialysed preparations were also stable at 27° over the period tested (12 hr.). When fresh preparations were allowed to stand at room temperature they increased in activity faster than similar preparations left at 2°; but the final activity was less.

At constant enzyme concentrations, the activities of enzyme extracts increased with increasing substrate concentration, to 0.005M-Dopa (Fig. 2). The optimum pH value for enzyme activity in 0.01M-phosphate buffer was pH 7.0.

Substrate specificity of Dopa oxidase

The Dopa oxidase was tested for action on various possible substrates. It oxidized L-tyrosine and *p*-cresol with lag periods, Dopa and pyrocatecho rapidly and *p*-phenylenediamine very slowly; it did not oxidize hydroquinone.

Presence of cytochrome and oxidase in mycelia of various ages

The visible absorption bands were at approximately 603, 564, 550 and 518 $m\mu$., corresponding to the cytochrome bands (*a*₃), *b*, *c* and *d* of yeast at 603, 564, 549 and 519 $m\mu$. respectively (Keilin, 1925). On passing O₂ gas or oxidising suspensions with a drop of H₂O₂, the bands faded and were replaced by a diffuse and weak band at *c*. 566 $m\mu$.

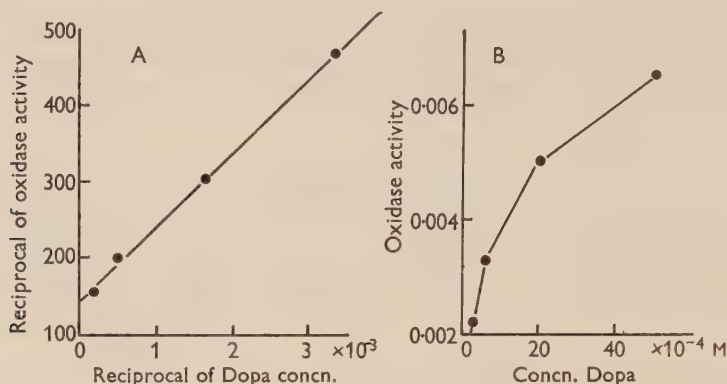


Fig. 2. The effect of DL-3:4-dihydroxyphenylalanine concentration on the activity at constant Dopa oxidase concentration. Extract prepared as in text. Spectrophotometer cells of 1 cm. light path contained in a total vol. 3.0 ml.; 10 μ g. extract N; Dopa concentration as indicated and 0.01 M-phosphate buffer (pH 7.0). Activity expressed as Δ o.d./10 sec./10 μ g. extract N.

The bands could be seen *in situ* in reduced mycelial felts up to and including 3-4 days old. In older material, owing to brown pigment formation, they were not seen. Particle preparations were therefore made from the older mycelium, by the same method as for cytochrome oxidase determinations, except that 18% (w/v) of sucrose was included in the preparative and washing fluids. The washed particle preparations were resuspended in 70% (w/v) sucrose in water with a little sodium dithionite and examined spectroscopically. In this way, it was possible to establish the presence of the cytochrome system at all mycelial ages up to 7 days.

Table 5. *The cytochrome c oxidase and Dopa oxidase activity of extracts prepared from mycelia of various ages*

Extracts prepared and activity assayed as in text.

Age of mycelium in days	Cyt. <i>c</i> oxidase activity (μ l O ₂ uptake/hr./mg. N)	Dopa oxidase activity (Δ o.d./min./mg. N)
1	1500	0
1½	1950	0
2	1900	0.4
3	1760	4.7
4	1600	4.0
5	1200	4.0

Assays for cytochrome *c* oxidase and Dopa oxidase activity of extracts prepared from mycelia of various ages showed that, while cytochrome *c* oxidase activity remained approximately constant with age of mycelium, Dopa oxidase activity was undetected before 48 hr. (Table 5). Maximum specific activity of Dopa oxidase was found with extracts from 3-day mycelium and the activity of extracts from 4- and 5-day mycelia was approximately constant. Undialysed extracts of young mycelia showed no Dopa oxidase activity.

Effects of some respiratory inhibitors on the activity of the extracted enzymes

Cytochrome *c* oxidase was inhibited by CO in the dark and by KCN but not by phenylthiourea. Dopa oxidase was inhibited by CO in the light and in the dark, by KCN, phenylthiourea and sodium diethyldithiocarbamate (Table 6).

Table 6. *Effects of some respiratory inhibitors on the cytochrome c oxidase and Dopa oxidase activity of cell-free extracts prepared from the mycelium*

For cytochrome *c* oxidase activities, Warburg manometer flasks contained in a total of 2.5 ml.: 0.5 ml. extract; 0.01 M-phosphate buffer (pH 7.2) containing 0.001 M-Versene; 50 μ M-cytochrome *c*; 0.1 M-hydroquinone in side arm and 0.15 ml. (w/v) KOH in centre well. Gas phase: air or as indicated; temp. 25°. Extract from 36 hr. mycelium.

For Dopa oxidase activities, Warburg manometer flasks contained in a total of 2.0 ml.: 0.5 ml. extract; 0.005 M-Dopa; 0.01 M-phosphate buffer (pH 7.0); 0.15 ml. 15 % (w/v) KOH in centre well. Gas phase: as indicated; temp. 25°. Spectrophotometer cells of 1 cm. light path contained in a total volume of 3.0 ml.: 0.2 ml. extract; 0.005 M-Dopa and 0.01 M-phosphate buffer (pH 7.0). Inhibitors (as in table) added to comparable samples. Extract from 4-day mycelium.

Details of extracts and assays in text.

Enzyme system	Inhibitor	Inhibition (%)
Cytochrome <i>c</i> oxidase	17:1, N ₂ :O ₂ } Dark	0
	17:1, CO:O ₂ }	70
	17:1, N ₂ :O ₂ } Light	0
	17:1, CO:O ₂ }	0
	0.001 M-KCN	95
	Sat. phenylthiourea	0
Dopa oxidase	17:1, N ₂ :O ₂ } Dark	0
	17:1, CO:O ₂ }	60
	17:1, N ₂ :O ₂ } Light	0
	17:1, CO:O ₂ }	60
	0.001 M-KCN	75
	Sat. phenylthiourea	100
	0.001 M-dieca	65

Effects of the inhibitors on the endogenous respiration of intact mycelium

In order to measure the light-reversible inhibition of respiration by CO, the O₂ uptake of samples of washed mycelium were determined in the presence and absence of CO in the light and in the dark. Mycelia of all ages had an endogenous activity which was not greatly affected by the presence of glucose; glucose was therefore omitted from the assay medium in later experiments. The inhibition was determined relative to control samples respiring in air; also included in Table 7 are the results of experiments with samples in N₂/O₂ mixtures. Since the CO inhibition was competitive with respect to O₂, it was

necessary to use a high CO:O₂ ratio; however, very low proportions of O₂ caused inhibition of the respiration and the ratio of 17:1 (v/v) CO or N₂ to O₂ was therefore chosen. Even with 17:1 mixtures, it was necessary (a) to have growth conditions such that the mycelium grew as fine strands, and (b) not to have more than 2 mg. dry wt. of 24-hr.-old mycelium, increasing to 8 mg. at 4 days old, if the lowered O₂ tension was not to cause inhibition of O₂ uptake when compared with rates in air. Under these conditions, there was no significant difference between the rates in air and the rates in 17:1 (v/v) N₂:O₂ except at 2½ and 3 days, when samples respiring in the N₂/O₂ mixture were slightly inhibited. The CO inhibition was completely reversed by light, except at 2½ days, when about 10 % remained (Table 7).

Table 7. *Inhibition of the respiration of intact mycelium of different ages in 17:1 (v/v) CO:O₂ mixtures, in the light and in the dark*

Warburg manometer flasks contained in a total volume of 2.0 ml.: 2–8 mg. dry wt. mycelium; 0.01 M-phosphate buffer (pH 6.0); 0.15 ml. 15 % (w/v) KOH in centre well. Gas phase: as indicated; temp. 25°.

Age of mycelium in days	Q _{O₂} μl./hr./mg. dry wt.		$\frac{Q_{O_2}(\text{air}) - Q_{O_2}(\text{CO:O}_2)}{Q_{O_2}(\text{air})} \times 100 \%$	
	Air	N ₂ :O ₂	Light	Dark
1	58	59	0	65
1½	50	49	0	60
2	36	38	0	60
2½	30	26	Variable about 20	65
3	27	23	10	60
4	20	22	0	60
5	19	—	—	—
6	17	18.5	—	—

0.001 M-KCN inhibited the respiration of 3½-day mycelium 80–90 % while phenylthiourea was without effect.

DISCUSSION

Darby & Goddard (1950*a*) showed that with standard conditions of inoculation, culture and assay, it was possible to obtain reproducible results in respiration studies with fungal material. This has been confirmed with *Gelasinospora tetrasperma*. Cytochrome oxidase is the major terminal oxidase in the respiration of this organism. This enzyme was extracted from the mycelia of various ages and the light-reversible CO inhibition of its activity was 70 %. With similar conditions, the CO inhibition of the respiration was about 60 % and, except with 2½-day mycelia, it was completely reversed by light. If therefore the extracted enzyme was inhibited to the same extent when it was within the mycelium, the cytochrome system was responsible for approximately 85 % of the O₂ uptake of the mycelium. This was confirmed by using cyanide, which inhibited the extracted enzyme almost completely and the respiration 80–90 %. These results are in contrast with those of Darby &

Goddard (1950*b*) who extracted cytochrome oxidase from the mycelium of *Myrothecium verrucaria*; this enzyme was inhibited by CO and KCN yet the respiration was unaffected by these inhibitors.

A Dopa oxidase was extracted from 2- to 5-day mycelia and characterized as tyrosinase by its substrate specificity and inhibition with CO in the light and in the dark. This enzyme was more or less inactive *in situ* since the CO inhibition of the respiration was completely reversed by light, apart from 10 % of that of 2½-day mycelia.

Horowitz & Shen (1952) showed that a dialysable inhibitor caused inactivity of the tyrosinase of *Neurospora crassa* when *in situ*. This is not so, however, with *Gelasinospora tetrasperma* since undialysed extracts when added to dialysed extracts did not inhibit their activity.

Tyrosinase activities of extracts tested immediately on grinding the mycelium were considerably less than when stored for a period under suitable conditions, suggesting that the enzyme was in an inactive form within the mycelium. A similar conclusion has been reached by Mallette & Dawson (1949) and Mason (1956) who found that changes in the enzyme took place on purification which led to an increased diphenolase activity. Singer & Kearney (1954) pointed out that these changes may have occurred during extraction rather than purification.

Lastly, it may be that the inactivity of the enzyme when *in situ* is partly due to conditions within the mycelium such as non-availability of substrate or low O₂ tension (Nelson, 1950).

The small proportion of the respiration unaffected by KCN or CO may be explained by the presence of an enzyme system insensitive to these poisons; this system might only function when the metal-containing enzymes have been inhibited (James, 1953*a*).

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The Nutritional Requirements of *Clostridium perfringens*

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SUMMARY: The requirements for growth factors, amino acids and inorganic constituents were determined for three strains of *Clostridium perfringens* (Veillon & Zuber) Holland. A number of substances were tested as energy sources for this organism, and the influence of pH value and temperature on growth was determined. The minimal medium evolved contained: alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, leucine (methionine), phenylalanine, threonine, tryptophan, tyrosine and valine; ammonium chloride, magnesium chloride, ferrous chloride, sodium-potassium phosphate buffer; glucose; adenine, biotin, calcium pantothenate and pyridoxine.

For maximal growth the presence of lysine, glycine and serine was necessary. Maximal growth was affected by the balance of amino acids in the medium; the balance of sodium and potassium ions was also important. Certain strain differences were noticed with respect to amino acid and vitamin requirements. Methionine was needed by one strain only; none of the strains required serine (reported essential for strain BP6K of Boyd, Logan & Tytell, 1948*b*), whereas aspartic acid was essential for all strains tested but not for BP6K. Riboflavine, an essential growth factor in BP6K, had no effect on growth of the strains tested. One of the strains showed a need for added nicotinamide when transferred to a nicotinamide-free medium after several transfers in peptone water.

In an investigation of the sulphur utilization of *Clostridium perfringens* (Veillon & Zuber) Holland, a chemically defined medium was needed, and a study of the nutritional requirements was therefore undertaken. Bacteria belonging to the genus *Clostridium* are usually grown on complex media including beef-infusion broth, glucose + peptone, and tryptic-digested casein, often with added liver or yeast extract. In investigations so far carried out on the nutritional requirements of this group, *C. sporogenes*, *C. tetani* and the butyl alcohol-producing clostridia have been given prominence.

The toxin production of *Clostridium perfringens* has been studied by several investigators and for this purpose many simplified media have been described (Taylor & Stewart, 1941; Reed & Orr, 1941; Robertson & Keppie, 1941; Stewart, 1942; Adams & Hendee, 1945; Logan, Tytell, Danielson & Griner, 1945; Tamura, Tytell, Boyd & Logan, 1941). However, all these media are based upon enzymically digested casein, meat-infusion or peptones with addenda such as meat or liver extracts.

Growth of *Clostridium perfringens* in a defined medium was first reported by Gladstone, Fildes & Richardson (1935) who used a mixture of amino acids, inorganic salts, glucose and mercaptoacetic acid, with the addition of a preparation from mare urine (later, of yeast) the 'sporogenes vitamin' of Knight & Fildes (1933). Shull & Peterson (1948) showed that the activity of

'sporogenes vitamin' preparation was probably due to its containing biotin and *p*-aminobenzoic acid. Pappenheimer & Shaskan (1944), in a study of the role of iron in the carbohydrate metabolism of *C. perfringens*, obtained growth in a casein-hydrolysate Tryptone (Difco) medium and in a partially defined medium based on hydrolysed purified gelatin with added amino acids, glucose, inorganic salts, and known growth factors, but only with regard to iron were the requirements studied more closely. In the course of their investigations on the toxin production of *C. perfringens*, Boyd, Logan & Tytell (1948*a*) studied the growth requirements more closely with special reference to vitamin and amino acid requirements, and a defined medium was described.

With our strains certain differences in the amino acid and vitamin requirements as compared with the findings of Boyd *et al.* were observed. Experiments were therefore carried out on the nutritional requirements of several strains and extended to examine also the inorganic constituents and energy sources. The present paper reports the results of this work on three different strains of *Clostridium perfringens*.

METHODS

Organisms. All the strains used were isolated in sulphite glucose iron agar (Wilson & Blair, 1924) from rotting tank sludge obtained from the Municipal Sewage Treatment Works at Copenhagen. The samples of sludge were pasteurized (80°, 5 min.) before incubation, and black colonies which developed were identified by microscopy, fermentation of sugars, and the stormy fermentation reaction in skim milk.

Cultivation. Experiments were carried out in test tubes containing 5 ml. medium. After inoculation the tubes were evacuated by means of an oil pump, sealed (see Lebert, 1949) and incubated at 37° for 20 hr. In experiments where the requirements for micronutrients were determined, special test tubes of Pyrex or Jena glass were used and incubated in anaerobic jars. Control experiments showed that growth was identical with both methods.

Growth was estimated by reading optical densities of the cultures with a Lumetron photoelectric colorimeter Model 402E, later by a Beckman colorimeter Model C with a red filter, with uninoculated culture medium as the blank. The Beckman colorimeter had the advantage that selected Pyrex culture tubes could be used as cuvettes, making it possible to follow anaerobic growth continuously in the same tube. The nitrogen content of a concentrated washed bacterial suspension was determined by the micro-Kjeldahl method, and the optical density of a sample was measured in a series of dilutions. Within the range of optical density of 0.14 to 0.65 scale units the increase in optical density was found to be linear with the nitrogen content. The optical density observed was converted to $\mu\text{g. N/ml. medium}$ by a multiplication constant $k = 100.8$.

Preparation of inoculum. Stock cultures were grown anaerobically in peptone water and maintained by daily transfers. After incubation for 15 hr. the organisms were centrifuged down, washed and resuspended in the buffered salt

solution used in the preparation of the medium. In experiments on the inorganic requirements, the constituent under investigation was omitted from the wash solution. The nitrogen content of the inocula varied between 5 and 20 $\mu\text{g. N/ml.}$, the size of the inoculum within this range being without influence on the amount of growth obtained; as inoculum 0.1 ml. washed suspension was used per 5 ml. medium.

Preparation of media. The composition of the media used is given in Table 1. The substances numbered from 1 to 5 constituted the buffered salt solution used as wash solution.

The pH value of all media was adjusted to 7.0–7.2 with sodium hydroxide except for sodium-free medium where potassium hydroxide was used. The pH value for routine use was determined with indicator papers (Special Indicators, Merck); in other cases electrometrically (Radiometer). The media with the omission of the constituent under investigation were pipetted into culture tubes and autoclaved at 110° for 10 min.

All the reagents used were of analytical grade (Merck); the amino acids were obtained from Hoffmann, La Roche and Co., the water used was double-distilled.

RESULTS

Effect of inoculum, pH value and temperature

Luxuriant growth occurred in both media 1 and 2. Because of the appreciable difference in cost the partially defined medium 2 was used in all preliminary experiments for determining the optimal pH value, temperature and incubation time, also in preliminary experiments on growth factor requirements and carbon utilization. Medium 1 was used in the final determinations of the basal requirements, in investigations concerning the nitrogen requirements, and with certain modifications in evaluating the need for inorganic constituents. Twice the concentration of sodium and potassium salts was originally used, but when the sodium requirements had been determined, we changed to media 1 and 2.

In determining whether a substance is essential for growth of bacteria two different methods are available: (1) subcultivation in a medium lacking the constituent under examination until growth eventually ceases; (2) washing the bacteria carefully before inoculation in order to remove traces of growth factors and micronutrients which might be carried over with the inoculum. Preliminary experiments indicated that with the latter method reliable results are obtained and much time saved.

Different washing procedures were tried, using sterile distilled water, 0.8% sodium chloride, or the buffered salt solution (1 to 5) of medium 1. With unwashed organisms as inoculum growth could be observed by naked eye 4 hr. after inoculation, and maximum growth was attained within 10 hr. When physiological saline was used for washing the lag period was prolonged, growth not being observable in the 11 hr. following inoculation, maximum growth nevertheless being obtained within 20 hr. With buffered salt solution as washing medium growth was retarded as compared with unwashed organisms, but in this case growth was evident within 6–8 hr.; 10 hr. after inoculation almost

Table 1. *The composition of the different media used*

Number of medium ...	1, defined medium	2, partially defined medium	3, minimal medium	4, basal medium	5, phosphate- free medium	6, sulphur- free medium
1. $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (g.)	6	6	6	6	—	—
2. KH_2PO_4 (g.)	1.5	1.5	1.5	1.5	—	2.27
3. NH_4Cl (g.)	1	1	1	1	1	1
4. NaCl (g.)	2.5	—	2.5	2.5	—	—
5. MgCl_2 (g.)	0.1	0.1	0.1	0.1	0.1	0.1
6. FeCl_3 (mg.)	0.7	0.7	0.7	0.7	0.7	0.7
7. Sodium diethyl-barbiturate (0.1 N) (ml.)	—	—	—	—	500	500
8. HCl (0.2 N)	—	—	—	—	To pH 7.1	To pH 7.1
9. KCl (g.)	—	—	—	—	1.64	—
10. Glucose (g.)	4	4	4	4	4	4
11. Adenine. HCl (mg.)	10	10	10	10	10	10
12. Biotin ($\mu\text{g.}$)	0.4	0.4	0.4	0.4	0.4	0.4
13. Calcium pantothenate ($\mu\text{g.}$)	200	200	200	200	200	200
14. Pyridoxine ($\mu\text{g.}$)	800	800	800	800	800	800
15. Further growth factors	†	†	—	—	—	—
16. Ascorbic acid (mg.)	5	—	5	5	5	5
17. Mercaptoacetic acid (ml.)	—	0.3, 80 %	—	—	—	—
18. Bacto vitamin-free Casamino acids	—	13.7†	—	—	—	—

19. Amino acids

	mg./l.§		mm§	mg.	mg./l.		mm	mg./l.		mm	
L-Tryptophan*	180	0.9		10	10	0.05		10	0.05		
DL-Alanine**	560	6.3		—	45	0.5		140	1.57		—
L-Arginine*	410	2.35		—	90	0.5		102	0.6		—
L-Aspartic acid*	630	4.73		—	65	0.5		160	1.2		—
L-Cystine*	40	0.17		—	120	0.5		240	1.0		—
L-Glutamic acid*	2280	15.5		—	95	0.5		500	3.4		—
Glycine	50	0.65		—	—	—		500	6.6		—
L-Histidine.HCl*	304	1.6		—	95	0.5		100	0.5		—
DL-Isoleucine**	650	5.0		—	65	0.5		150	1.1		—
L-Leucine*	1210	9.23		—	65	0.5		300	2.2		—
L-Lysine.2HCl	930	4.3		—	—	—		500	2.3		—
DL-Methionine	350	2.37		—	—	—		100	0.6		—
L-Hydroxyproline	200	1.52		—	—	—		—	—		—
L-Proline	820	7.1		—	—	—		—	—		—
DL-Phenylalanine*	520	3.15		—	80	0.5		130	0.8		—
DL-Serine	750	7.1		—	—	—		500	4.7		—
DL-Threonine*	390	3.8		—	60	0.5		100	0.95		—
DL-Tyrosine*	640	3.15		—	90	0.5		160	0.8		—
DL-Valine*	700	6.0		—	60	0.5		175	1.5		—

* The indispensable amino acids are marked with an asterisk.

** Not strictly indispensable, but strongly stimulating amino acids.

† Further growth factors used in preliminary experiments: guanine (10 mg.), uracil (10 mg.), xanthine (10 mg.), *p*-aminobenzoic acid (200 µg.), nicotinamide (1 mg.), riboflavin (100 µg.), thiamine (20 µg.), vitamin B₁₂ (0.02 µg.), folic acid (0.1 µg.); pimelic acid (200 µg.).

‡ This amount contains, according to the manufacturer (Difco), about 0.9 g. amino-N and 5 g. NaCl besides other inorganic salts, hence the NaCl added to other media was omitted from medium 2.

§ The amino acid composition of the medium 1 is chosen to correspond approximately to 1 % casein according to the data of Block & Bolling (1947).
|| The amino acid composition of the phosphate-free medium and the sulphur-free medium is the same as in the minimal medium.

maximal growth was obtained. When the organisms were washed with distilled water the results were variable and often only slight growth occurred.

Addition of reducing agents (e.g. mercaptoacetic acid) in low concentrations to the wash solutions did not improve the growth rate obtained; with higher concentrations a marked prolongation of the lag phase was observed.

The rate of growth also markedly depended on the age of the culture, where-as the number of bacteria in the inoculum, within the range used (equivalent to 2–20 $\mu\text{g. N/ml.}$) was without influence on the maximal growth. With a 4 hr. culture as inoculum, washed in the buffered salt solution, maximum growth was obtained within 6 hr.; with a 15 hr. culture growth became evident within 6 hr., but the maximum growth was not reached until 10–18 hr.; with a 20 hr. culture growth did not begin before 10 hr., with maximal growth within 20 hr.

In the defined medium autolysis started soon after 25 hr. of incubation, the turbidity decreasing rapidly.

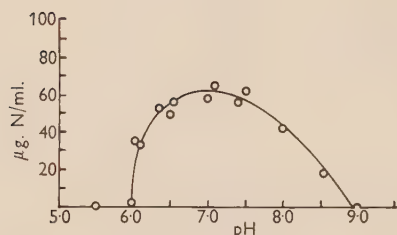


Fig. 1. The effect of pH upon the growth of *Clostridium perfringens*.

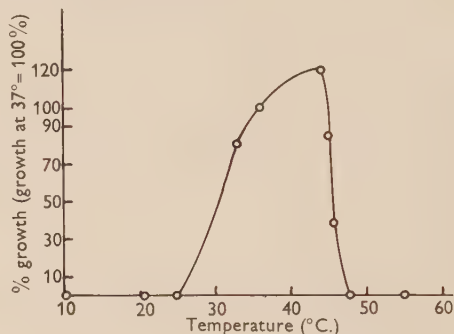


Fig. 2. The dependence of the growth of *Clostridium perfringens* on temperature (Growth at 37° = 100 %.)

Boyd *et al.* (1948*a*) reported an acceleration of growth by adding adenine, guanine or uracil, maximum growth occurring also in a medium without added purines, although at a slow rate (within 25 hr.). The strains tested in the present work showed an absolute requirement for adenine, not even retarded growth occurring in its absence or when it was replaced by the other purines.

Gladstone *et al.* (1935) stated that carbon dioxide is an essential factor for certain bacteria including *Clostridium perfringens*, and that carbon dioxide must be present in all media before multiplication begins. In the present work added carbon dioxide was not necessary for the growth of the *C. perfringens* strains used. In all cases maximal growth was obtained within 20 hr.

The optimal pH value of the medium was determined, the relationship between growth and pH value being shown in Fig. 1; optimal growth was obtained between pH 6.75 and 7.5.

The growth-temperature relationship was determined for a strain which had been maintained in laboratory media at 37° for 10 weeks. The tubes were incubated at ten different temperatures in medium 2. The results are shown in Fig. 2; optimal growth occurred between 37° and 44°.

Neither medium 1 nor 2, which include the growth factors, supported growth unless a reducing agent was added; mercaptoacetic acid, cysteine or ascorbic acid were effective; thioacetamide and sulphide were ineffective. Better growth was obtained with mercaptoacetic acid than with ascorbic acid; except when a sulphur-free medium was desired, mercaptoacetic acid was generally used.

Vitamin requirements

No growth occurred in medium 1 or 2 with added ferrous ions and mercaptoacetic acid when the other accessory factors were omitted, but the addition of a group of known growth factors, as listed in Table 1, resulted in heavy growth. The accessory factors were then individually omitted from the medium in a series of experiments.

The following were found to be necessary for the growth of the strains of *Clostridium perfringens* tested (concentrations for optimal growth in brackets; $\mu\text{g./ml.}$): adenine (10); calcium pantothenate (0.2); pyridoxine (0.8); biotin (0.00004).

The growth obtained in medium 1 or 2 in the presence of these growth factors was as heavy as that obtained in meat extract broth + ferrous ions + mercaptoacetic acid or in peptone + glucose. Riboflavine (reported to be essential for strain BP6K by Boyd *et al.* 1948*a, b*), did not have any effect on the growth of the strains tested, neither did the addition of other growth factors have any stimulatory effect. The growth response to adenine, calcium pantothenate, pyridoxine and biotin was similar to that described by Boyd *et al.* Adenine, calcium pantothenate and pyridoxine were indispensable for all strains studied. Some growth occurred in a medium lacking added biotin, but the addition of biotin was then greatly stimulating. Some lots of the Bacto Vitamin-free Casamino acids contain traces of adenine or some substance equivalent to adenine, occasionally also traces of B-vitamins and tryptophan, because some growth occurred even without the addition of these factors, although the same culture in the completely defined medium 1 did not grow unless these substances were added.

Nicotinic acid or nicotinamide was not required by freshly isolated strains and did not have any stimulatory effect, but after many passages in laboratory media rich in nicotinic acid (peptone water) some strains grow much better when nicotinamide or nicotinic acid was added to a nicotinamide-free medium. These strains also lost their sulphite-reducing capacity in a medium which lacked nicotinamide.

Inorganic requirements

Following the determination of the basal physical and chemical requirements of the culture medium the effect of the balance of the chemicals used was investigated.

Sodium and potassium. The effect of sodium and potassium ions in varying concentrations was studied. The sodium-free medium consisted of the minimal medium, number 3 in Table 1, but with the sodium-potassium phosphate buffer and the sodium chloride replaced by a buffer prepared with primary

potassium phosphate, potassium hydroxide and potassium chloride; sodium was added as sodium phosphate. The potassium-free medium was prepared in a similar manner with a pure sodium phosphate buffer and sodium chloride; potassium was added as chloride. No special precautions were taken to remove traces of the two alkali metals present as impurities.

Table 2. *The dependence of growth of Clostridium perfringens on sodium and potassium concentrations*

A. Increasing sodium concentration with constant potassium concentration at different potassium concentrations.

Normality of Na	K=0.05 N	K=0.125 N	K=0.215 N
0	÷	÷	÷
0.0001	÷	÷	÷
0.001	÷	÷	÷
0.003	+	÷	÷
0.01	++	+	÷
0.1	++	++	+
0.25	÷	+	+
0.45	÷	(+)	(+)
0.83	÷	÷	÷

B. Increasing potassium concentration.

Normality of K	Na=0.119 N	Na=0.166 N
0	(+)	(+)
0.0001	++	++
0.0004	++	++
0.001	++	+
0.01	++	+
0.1	+	(+)

÷ denotes no growth; (+), +, ++ denotes increasing degrees of growth.

It is seen from the results (Table 2) that in their influence upon the growth of *Clostridium perfringens* in a defined medium, the concentrations of sodium and potassium ions were mutually dependent. Sodium was indispensable, but only traces of potassium were needed, some growth occurring without added potassium. Additional potassium, however, enhanced growth, and optimal growth was obtained with potassium concentrations varying from 0.0001 to 0.05 M; higher potassium concentrations depressed growth unless the sodium concentration was also increased. The threshold concentration of sodium changed from 0.03 to 0.1 M when the potassium concentration was increased from 0.05 to 0.215 M. On the other hand, growth was possible with potassium at 0.1–0.215 M with such sodium concentrations as would inhibit growth at lower potassium concentrations. The toxic concentration of sodium was thus raised from 0.25 to >0.45 M when the potassium was increased from 0.05 to 0.215 M. The optimal sodium concentration in the defined minimal medium (number 3 of Table 1) varied between 0.01 and 0.1 M, depending on the potassium level.

Phosphate. In experiments in which the requirement for phosphate was studied, a sodium diethylbarbiturate buffer was used (medium 5, Table 1). The pH value was adjusted with 0.2 N-hydrochloric acid. Phosphate was

indispensable for the growth of *Clostridium perfringens*; optimal growth occurred even with 0.00005 M-phosphate and the growth remained unaltered up to 0.125 M, after which it rapidly decreased.

Sulphate. It was not necessary to add either sulphate or any other compound to any of the media 1–4 (with mercaptoacetic acid replaced by ascorbic acid) for the growth of *Clostridium perfringens*. The question of the availability of sulphur is dealt with in another paper (Fuchs & Bonde, 1957).

Magnesium. Omission of magnesium chloride from the medium 1 or 2 resulted in complete lack of growth; this was also observed by Webb (1951) who obtained growth even with 1 mg./l.; optimal growth was obtained with our strains at 50–100 mg. MgCl_2 /l., the same concentration required for normal rod formation according to Webb; concentrations >100 mg./l. did not further increase growth.

Iron. Growth did not occur in a medium completely lacking in iron, provided that the inoculum organisms were well washed. Pappenheimer & Shaskan (1944) reported maximum growth at 0.6 mg. ferrous ion/l. medium. This was also the case with our strains; growth was not affected by increases beyond 0.6 up to 90 mg./l.

Other elements. When sodium and potassium phosphate, ammonium chloride, magnesium chloride and ferrous chloride were present in optimal amounts, the addition of other inorganic nutrients was superfluous. No increase in growth occurred when manganese, copper, zinc, and calcium were added singly or together.

Nitrogen requirements

The nitrogen required by *Clostridium perfringens* can be supplied by acid-hydrolysed casein, provided that L- or DL-tryptophan and an ammonium salt are added. The casein hydrolysate may be substituted by a mixture of 19 pure amino acids of approximately the same composition as 1% casein (based on the data of Block & Bolling, 1947), the growth being then as heavy as with casein hydrolysate (amino acids used listed Table 1). The concentration of the amino acids could be decreased to one-fourth without affecting growth.

Ammonium ion cannot be omitted from the medium; without added ammonia slight growth occurred in the defined medium 1, and some in medium 2 which probably contained traces of ammonia; 500 mg. ammonium chloride/l. is required for maximal growth.

Amino acid requirements

When the need for individual amino acids was investigated, some differences in the requirements for different strains were observed. It became evident that the balance of certain amino acids in the medium was of importance. By omission of amino acids one at a time and in groups from the medium 1, the following were found to be essential for all strains of *Clostridium perfringens* tested (growth was not observed in their absence): arginine, aspartic acid, cystine, glutamic acid, histidine, leucine, phenylalanine, threonine, tryptophan, tyrosine and valine. Cystine could partly be replaced by mercaptoacetic acid,

which could supply some of the sulphur needed for growth, but growth was much heavier with cystine.

The effect of methionine varied considerably. The omission of methionine had no effect with strain B, slightly depressed the growth of strain A, and resulted in very poor growth of strain C. The omission of isoleucine and alanine singly from medium 1 also resulted in poor growth except with strain B, with which growth was only depressed to some degree in an otherwise complete medium free from isoleucine. In the absence of both no growth occurred.

The essential amino acids, including methionine for the methionine-requiring strain, alanine and isoleucine, constitute the minimal medium for *Clostridium perfringens*, denoted by 3 in Table 1 together with the buffered salt solution of medium 1, glucose (4 g./l.), ascorbic acid (5 mg./l.), adenine (10 mg./l.), biotin (0.04 μ g./l.), calcium pantothenate (200 μ g./l.), and pyridoxine (800 μ g./l.). The proportions of the amino acids are important for

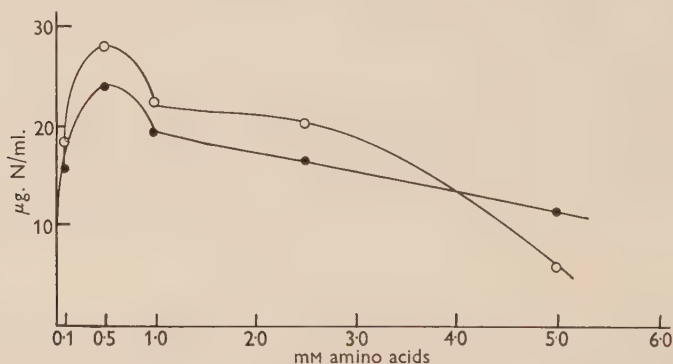


Fig. 3. The effect of increasing concentrations of amino acids on growth of *Clostridium perfringens*. The individual amino acids are present in equimolar concentrations, except L-tryptophan, which is only 0.05 mm. ●: indispensable amino acids; ○: indispensable amino acids + methionine.

maximal growth. In the mixture which approximates to 1% casein, where the acids are present in concentrations varying from 0.17 mm (cystine) to 15.5 mm (glutamic acid), the growth response was very good. When the acids were added in equimolar concentrations varying from 0.1 to 5.0 mm the growth response was maximal at 0.5 mm (Fig. 3); only half maximal growth occurred in the 5.0 mm medium.

The minimal amounts of amino acids which supported maximal growth in the complete medium of Boyd *et al.* (1948*b*) for strain BP 6 K were the following (calculated from the data of Boyd *et al.*, expressed in mmole/l.): L-arginine, 0.25; L-cystine, 0.083; L-glutamic acid, 0.81; L-histidine, 0.096; DL-isoleucine, 0.92; L-leucine, 0.38; DL-phenylalanine, 0.48; DL-threonine, 0.67; L-tryptophan, 0.39; L-tyrosine, 0.19; DL-valine, 0.13.

The minimal medium 3 of Table 1 supported growth of the three strains tested, well with strain A, rather poorly with strain C. Better growth in all cases was obtained in a medium which contained the indispensable amino

acids alanine, isoleucine and methionine in concentrations corresponding to 0.25% casein, the stimulatory amino acids glycine, serine and lysine being present in the high concentrations of Boyd *et al.* (1948*b*). This is the basal medium, number 4 in Table 1.

Antagonism between amino acids. Interrelationships between the remaining amino acids were noticed, indicating the existence of antagonistic effects similar to those observed with other organisms (Gladstone, 1939; Bonner, 1946; Doermann, 1944; Rowley, 1953; Washburn & Niven, 1948). In the case of *Clostridium perfringens*, however, the inhibitions did not seem to be specific, the action of certain amino acids being prevented by several other amino acids. The degree of inhibition varied also in the different strains, being most marked in strain C. Two groups were involved; glycine, lysine and serine, and hydroxyproline and proline.

In the minimal medium, number 3 in Table 1, the addition of glycine, lysine or serine improved growth, glycine having the greatest effect and lysine the smallest; hydroxyproline and proline had no effect. When added together, glycine+lysine had a synergistic effect; added serine depressed both the glycine and lysine effects. Hydroxyproline and proline were mutually dependent. With the medium containing the complete mixture of amino acids present in casein, the removal of proline resulted in considerable improvement in growth; following the omission of hydroxyproline growth remained unaltered, but the simultaneous omission of both depressed the growth obtained. The concentration of glycine, however, was low in the medium 1 (0.65 mmole/l.); when the concentration was increased to 6.6 mmole/l. the growth remained unaffected by the removal of the proline group from the medium. Maximal growth thus occurred in the basal medium, where the glycine concentration was high (6.6 mmole/l.) in the absence of the proline group.

The amino acid requirements determined by Boyd *et al.* for *Clostridium perfringens* strain BP6K differ in some respects from those found in the present work. As with our strains, arginine, cystine, glutamic acid, histidine, leucine, phenylalanine, threonine, tyrosine, valine and tryptophan were found to be essential, but also isoleucine, methionine and serine were absolute requirements of strain BP6K, whereas alanine and aspartic acid were not. However, they found that pyridoxamine or pyridoxal (or pyridoxine in amounts a thousand-fold larger) apparently eliminated the requirement of strain BP6K for lysine, alanine, aspartic acid and glycine. In the presence of pyridoxine in amounts which supported growth in the otherwise complete medium alanine and lysine were essential, and only very poor growth occurred in the absence of glycine or aspartic acid. The minimal amounts which supported maximal growth of BP6K in the otherwise complete medium were given in another paper by Boyd *et al.* (1948*b*). Only in the case of L-tryptophan was the optimal concentration determined in the present work. As shown in Fig. 4, 2.25 mg. L-tryptophan/l. were required by our strain A in medium 2 to obtain maximal growth, which is considerably lower than the concentration required for BP6K (8.0 mg./l.).

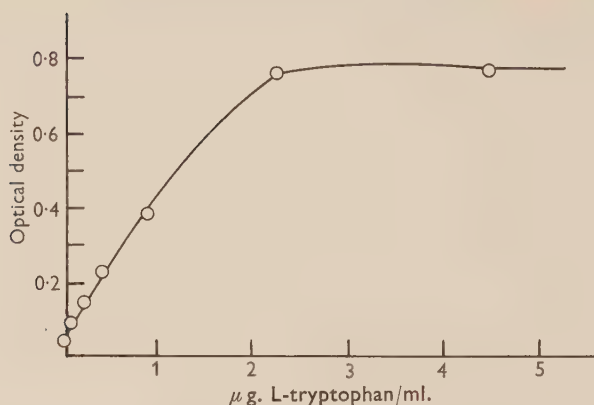


Fig. 4. Growth response of *Clostridium perfringens* to L-tryptophan in medium 2. Growth remains unaltered by increasing concentration until 180 µg./ml. At 450 µg./ml. the o.d. is 0.35 scale units.

Utilization of carbon compounds

Clostridium perfringens is strictly dependent on carbohydrates and similar compounds for its energy; no growth occurred in the casein hydrolysate medium 2 without addition of glucose. Other carbon compounds were tested as energy sources; only saccharides were utilized (see Table 3) and only galac-

Table 3. Growth of *Clostridium perfringens* in defined medium with different carbon compounds as substrates

Growth is expressed in percentage of the growth obtained with an equimolar amount of glucose.

Substrate	Growth (%)	Substrate	Growth (%)
Polysaccharides		Hexoses	
Inulin	12.6	Glucose	100
Soluble starch	72.4	Galactose	104
Disaccharides		Fructose	71.0
Saccharose	88.5	Pentoses	
Maltose	72.4	Xylose	20.6
Lactose	66.0	Arabinose	5.3
		Polyalcohol	
		Mannitol	0.6

The following compounds gave no growth: formate, acetate, propionate, butyrate, citrate, succinate, fumarate, pyruvate, lactate, gluconate, tartrate, glycerol, acetone, ethanol. Glycollate gave 7% and glycerophosphate 5% of growth in the units used.

tose gave as good growth as glucose. Sodium glycerophosphate, sodium glycollate and glycerol gave only slight growth. The results are summarized in Table 3. Sodium pyruvate had a growth-stimulating effect in the presence of glucose, although it alone did not support growth. Some other substances tested had an inhibitory effect in the presence of glucose; these were (in order of decreasing effectiveness): citrate, acetate, gluconate, succinate and formate.

Our sincere thanks are due to Professor P. Bonnevie, Director of this Institute, for valuable support and encouragement.

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The Availability of Sulphur for *Clostridium perfringens* and an Examination of Hydrogen Sulphide Production

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SUMMARY: The utilization of several inorganic and organic sulphur compounds by three strains of *Clostridium perfringens* and the production of hydrogen sulphide from these compounds was investigated. Sulphate, sulphite, thiosulphate and sulphide cannot supply sulphur to the organism, neither do they have any effect, when added in non-toxic concentrations, on the growth in media containing utilizable organic sulphur. Cystine or cysteine, which can be replaced by glutathione, were required by the three strains tested; no additional sulphur source was needed by two of the strains, the third requiring also methionine, which can be replaced by homocyst(e)ine. Hydrogen sulphide is produced by growing cultures from sulphite, thiosulphate, cystine, cysteine and glutathione, but not from methionine. Different enzyme systems are concerned with hydrogen sulphide production from cystine and sulphite, respectively. The sulphite-reducing capacity of some cultures was often decreased after several passages in laboratory media, the presence of nicotinamide being then required for this reduction. Sulphite was reduced to sulphide by suspensions of resting organisms. The presence of a hydrogenase system is suggested by preliminary experiments, the organisms being able to activate molecular hydrogen for reduction of methylene blue and sulphite. Other hydrogen donors are utilized by the organisms, in particular glucose. The addition of sulphites does not induce growth on substrates which do not support growth in sulphite-free media.

Hydrogen sulphide is produced by micro-organisms from compounds containing organic sulphur and from inorganic sulphur compounds, e.g. thiosulphate, sulphite, sulphate. Many different bacteria produce hydrogen sulphide from peptones, cystine and cysteine (Clarke, 1953; Stekol & Ransmeier, 1942), and some from thiourea (Tanner, 1917), but not from taurine (Sasaki & Otsuka, 1912; Bürger, 1914). A few produce H_2S from thiosulphate (Tanner, 1917; Tilley, 1923*a, b*) and from sulphite (Tilley, 1923*a, b*; Wilson, 1922-3; Tanner, 1917). Only a small group of sulphate-reducing bacteria (vibrios) has so far been described, first by Beijerinck (1895) and later studied more closely by several investigators.

Clostridium perfringens is capable of reducing sulphite in suitable media and forms colonies black with precipitated ferrous sulphide in iron sulphite agar (Wilson & Blair, 1924, 1927). The occurrence of *C. perfringens* in sewage before and after treatment in purification plants is used at this Institute as an indication of the effectiveness of purification, together with the coliform count. The isolation and counting is based on the sulphite-reducing capacity of these bacteria (Bonde & Hvid-Hansen, 1953). The mechanism of sulphite reduction, as well as that of sulphate and thiosulphate reduction, is still obscure, and it is also unknown what, if any, the significance of this reaction is to the bacterial metabolism. The aim of the present work is to investigate the conditions governing the reduction of sulphite by *C. perfringens*.

The studies of Roberts, Abelson, Cowie, Bolton & Britten (1955) on the sulphur metabolism of *Escherichia coli* showed that the total sulphur content of the bacterium depends in a large measure upon the sulphur compounds present in the chemically defined medium and that the utilization of sulphur by the organism from any particular sulphur source depends upon the chemical form of this sulphur compound and the type of organism used. The first part of the present paper reports the results of studies on the utilization of various sulphur compounds by *Clostridium perfringens*; the second part is concerned with hydrogen sulphide production from sulphite and other sulphur sources, and with the effect of the environment upon the reduction of sulphite.

METHODS

Organisms. All the strains used in this work were isolated from rotting tank sludge obtained from Municipal Sewage Works of Copenhagen. The general methods used for isolation, cultivation and the handling of inocula are described elsewhere (Fuchs & Bonde, 1957).

Standardization of suspensions. Amount of growth was measured as the turbidity of the cultures estimated by a Beckman colorimeter model C with a red filter, using uninoculated medium as a blank. It has previously been ascertained, that optical density of the cultures in the media used is, within certain limits, directly proportional to the nitrogen content of the suspension, as measured in a sample by the micro-Kjeldahl method (Fuchs & Bonde, 1957).

Medium. The composition of the media used is given in Table 1 of Fuchs & Bonde (1957, see p. 320 of this issue).

In the experiments concerning sulphur availability the minimal medium number 3 was generally used. The sulphur-containing amino acids were omitted when necessary. A sulphur-free medium, number 6, where the concentration of sulphate as impurities was minimized, was used to confirm the results obtained with the minimal medium about the effect of inorganic sulphur compounds on growth. In the medium 1 growth was heavier than in the minimal medium; it was therefore used to cultivate larger amounts of organisms and to confirm the results obtained with the minimal medium. Medium 2 (Fuchs & Bonde, 1957) was generally used in the experiments concerning sulphite reduction.

Medium was distributed in test tubes in 5 ml. amounts, autoclaved for 10 min. at 110° (with the ascorbic acid medium care must be taken not to exceed the temperature or time given), cooled, inoculated and incubated anaerobically according to the method of Prévot (see Lebert, 1949).

Sulphide detection. Numerous indicators are used to detect hydrogen sulphide production by micro-organisms, usually iron, lead, bismuth, nickel and cobalt salts. The sensitivity of these indicators is different in different media, and the results are not strictly comparable, as pointed out by Clarke (1953) and others (e.g. Hunter & Crecelius, 1938). In the present work two different methods were used.

(1) Iron salts. Ferrous ammonium sulphate or ferrous chloride were added in equimolar concentrations with the added sulphite. This method is not very

sensitive. In our media sulphide produced from cystine or other organic sulphur sources or from $1\text{ }\mu\text{mole}$ sulphite/ml. was not detected by this means, the presence of $2\text{ }\mu\text{mole}$ sulphite/ml. was necessary for the formation of the black ferrous sulphide precipitate.

(2) Lead acetate paper strips were generally used to detect sulphide formed during growth. Before turbidity determination the ampoules were punctured while holding a moist strip of lead acetate paper over the small opening. When the tubes were incubated in anaerobic jars, the strip was inserted in the mouth of the tube and held by a sterile cotton plug. It was noticed that uninoculated media containing mercaptoacetate liberated some hydrogen sulphide during incubation at 37° . When sulphide formation from unknown sulphur sources or by unknown cultures has to be detected, the media must not contain mercaptoacetate.

The lead acetate paper method is much more sensitive than the use of iron salts, strong blackening of the paper occurring with our media in cultures where no ferrous sulphide formation yet could be detected. ZoBell & Feltham (1934) reported, in the media used by them, a 10 to 100 times greater sensitivity of lead acetate as compared to iron and bismuth salts. By combining both methods it is possible to compare approximately the amounts of hydrogen sulphide formed without quantitative measurements. Such amounts of sulphide which cause a blackening of the lead acetate paper too intense to grade, will give rise to ferrous sulphide precipitate. With lower sulphide concentrations, below the sensitivity of iron salts, a rough grading of the degree of blackening of the lead acetate paper is possible.

Studies with washed bacterial suspensions. For studies of the bacterial enzymes which catalyse the reduction of sulphite, suspensions of resting organisms were prepared by cultivation in the medium 2 with added nicotinamide, in volumes of 100–300 ml., incubated in evacuated flasks for 16–20 hr., centrifuged, washed twice in 10–20 ml. of the buffered salt solution (1–5) of media 1 and 2 and resuspended in 10–20 ml. of this solution. These suspensions lost their reducing capacity rapidly, even when stored in the icebox under hydrogen or nitrogen and had to be used the day of preparation. In these experiments it was of great importance that the bacterial suspensions were prepared under as similar conditions as possible; the harvesting should be performed as soon as maximal growth is obtained.

The reducing capacity of the bacterial suspensions was studied by the Thunberg technique in evacuated ampoules, containing 1 ml. $0\cdot00025\text{ M}$ -methylene blue, 1 ml. $0\cdot1\text{ M}$ substrate, 1 ml. $0\cdot05\text{ M}$ -phosphate buffer (pH 7·0–7·2) and 1 ml. bacterial suspension.

Warburg vessels were used when methylene blue was replaced by sulphite; a strip of filter paper moistened with lead acetate was placed in the centre well.

RESULTS

Growth in sulphur-deficient media. The medium 1 (with omission of the sulphur-containing amino acids and mercaptoacetate; Fuchs & Bonde, 1957) did not support growth without added sulphur. A slight increase in the

bacterial cell mass occurred, just detectable by the colorimeter used, which corresponded to about 1 µg. N/ml. This increase must be ascribed to sulphur reserves of the organisms (see Roberts *et al.* (1955) with *Escherichia coli*), and not to impurities present in the reagents used, because added inorganic sulphur as sulphide, sulphite and sulphate did not improve growth.

Growth response to inorganic sulphur as sole sulphur source. Inorganic sulphur as ammonium sulphate, sodium sulphite, sodium thiosulphate, or sodium sulphide was added in varying concentrations to the sulphur-free media 1 or 6; no growth was obtained with any concentration of these salts. Thus *Clostridium perfringens* is unable to utilize these compounds as sole source of sulphur.

Table 1. *Growth response to organic sulphur-containing compounds of three different strains of Clostridium perfringens*

Sulphur compound	<i>C. perfringens</i>		
	Strain A	Strain B	Strain C
Thioacetamide	—	—	—
Mercaptoacetate	+	+	+
Cysteine	++	++	+(+)
Cystine	++	++	+(+)
Methionine	Trace	—	+
Homocysteine	—	—	+
Homocystine	—	Trace	+
Glutathione	+	—	+(+)
Taurocholate	—	—	—

Growth response to organic sulphur compounds. The sulphur-containing substances listed in Table 1 were tested as possible sulphur sources for three different strains of *Clostridium perfringens*. Some differences were observed in the response to different sulphur-containing amino acids among these strains; the results are summarized in Table 1. Strain A was kept in laboratory media for over a month before the experiments, strain B was a newly-isolated strain, and C had been maintained in laboratory media for over three months. Cystine, cysteine and glutathione were nearly equally good as a sole source of sulphur for all strains. Mercaptoacetate alone could also support some growth, while thioacetamide was unable to supply sulphur. Methionine, homocysteine and homocystine failed to support growth of strains A and B. With strain C, which requires both cystine and methionine for optimal growth, suboptimal growth occurred also with homocysteine or homocystine. The effect of increasing concentration of cysteine and cystine is shown in Table 2.

Effect of additional sulphur compounds on growth in cystine-containing media. When sulphate, sulphite or sulphide was added in concentrations varying from 0.1 to 500 µg. S/ml., no increase in growth could be obtained over that occurring in the controls containing cystine only. Sulphate did not have any inhibitory effect in the concentrations used, but sulphite and sulphide both depressed growth when the concentration was increased to 100 µg. S/ml. and more. Table 3 shows the effect of increasing concentrations of sulphate,

sulphite and sulphide on growth in cystine media. When adequate amounts of cystine were present the addition of cysteine, mercaptoacetate or thioacetamide was without effect on growth. Methionine had a stimulatory effect with strain A, little effect with strain B, but with strain C only very poor growth was obtained in the absence of methionine.

Table 2. *Growth response of Clostridium perfringens (strain A) to increasing amounts of cysteine and cystine*

Concn. of cysteine ($\mu\text{g./ml.}$)	Optical density (scale units)	Concn. of cystine ($\mu\text{g./ml.}$)	Optical density (scale units)
0.1	0.0	0.1	2.5
1.0	0.0	0.5	5.4
10.0	14.1	10.0	29.5
100.0	35.9	100.0	25.2
1000.0	0.8		

Table 3. *Effect of increasing concentrations of sulphate, sulphite and sulphide on growth of Clostridium perfringens (strain A)*

Sulphur added ($\mu\text{g. S/ml.}$)	Sulphur added as		
	Na_2SO_4	Na_2SO_3	Na_2S
	Optical density (scale units)		
0.0	21.2	21.2	21.2
0.1	.	18.1	21.6
1.0	18.1	21.6	19.4
5.0	19.4	.	.
10.0	21.1	21.6	21.2
50.0	22.3	19.6	.
100.0	20.8	10.6	16.2
200.0	21.6	.	.
500.0	23.8	1.3	0.0

The addition of homocystine to strains A and B in cystine medium had an inhibitory effect. With the methionine-requiring strain C, the addition of homocystine or homocysteine to cystine medium improved growth to nearly the same extent as methionine, but they could not replace cystine in methionine-containing medium.

Apparently methionine is synthesized from cystine (cysteine) by *Clostridium perfringens*, but not the reverse. Homocysteine and homocystine serve as intermediates or, more probably, they are transformed in some way into the real intermediates. The reaction $\text{cyst(e)ine} \rightarrow \text{R} \rightarrow \text{homocyst(e)ine}$ is the one blocked in the methionine-requiring strain. Tables 4 and 5 show the growth response of the strains A, B and C to the above-mentioned organic sulphur compounds.

Growth and hydrogen sulphide formation

Organic sulphur compounds. Growth of *Clostridium perfringens* in cystine-containing medium always showed sulphide production, a very common reaction in bacterial metabolism. Other sulphur-containing organic substances giving rise to hydrogen sulphide production by *C. perfringens* are mercapto-

acetate, cysteine; and in the methionine-requiring strain C homocystine and homocysteine, although to a lesser extent than cyst(e)ine; but not methionine. Glutathione did not give hydrogen sulphide. Apparently free cyst(e)ine must be present, before hydrogen sulphide can be formed.

Inorganic sulphur compounds. Although no growth occurred with sulphite or thiosulphate as sole sulphur source, these ions are nevertheless attacked by a growing culture of *Clostridium perfringens* with reduction to hydrogen sulphide. *C. perfringens* did not reduce sulphate.

Table 4. *Effect of cysteine and methionine on growth of three strains of Clostridium perfringens in cystine-free medium and in cystine medium*

Sulphur compound added	<i>Clostridium perfringens</i>		
	Strain A	Strain B	Strain C
	Amount of growth (optical density scale units)		
Methionine	3.8	4.1	0.5
Cystine	30.3	27.6	10.5
Cysteine	21.0	15.0	.
Mercaptoacetate + methionine	9.7	.	.
Cystine + methionine	43.9	26.7	38.0
Cystine + methionine + SO ₄	40.0	.	.
Cystine + cysteine	22.3	30.0	.
Cysteine + methionine	30.0	31.0	.

Table 5. *Effect of homocystine and homocysteine on growth of a methionine-requiring strain of Clostridium perfringens*

Sulphur compounds added	Strain C
	(optical density scale units)
Methionine + cystine	53.3
Methionine + homocysteine	10.3
Methionine + homocystine	14.7
Cystine + homocysteine	42.0
Cystine + homocystine	47.0
Homocysteine + homocystine	15.0

Conditions for sulphite reduction. A newly-isolated strain will grow abundantly on medium 2 and in the presence of sulphite and ferrous-iron produce a black precipitate of ferrous sulphide. The minimal concentration of sulphite required for the formation of this precipitate is 2 μ mole/ml., maximally 10 μ mole/ml. After several successive transfers in peptone broth the threshold concentration of sulphite needed for the ferrous sulphide formation is increased, and eventually the ability to form ferrous sulphide in this medium is often lost, though not with all the strains.

In meat extract + peptone agar with added sulphite and ferrous iron, black colonies were formed even by strains which no longer produced any detectable ferrous sulphide in the medium 2. Apparently a factor which is present in meat-extract agar is needed for the reduction of sulphite. This factor was found to be nicotinamide. When nicotinamide was added to medium 2 a

ferrous sulphide precipitate was again formed with the same threshold concentration of sulphite as for a newly-isolated strain. With strain A growth was also improved by the addition of nicotinamide; with strain C growth was unaltered. The tolerance for sulphite was also increased by the addition of nicotinamide with strains which required it for sulphite reduction. In Table 6 is shown the effect of nicotinamide on growth and ferrous sulphide formation by a nicotinamide-requiring strain (A) and by a strain (C) not requiring this factor. It is evident from the results in Table 6 that nicotinamide alone was responsible for the effect with strain A, the addition of various other growth factors being without effect.

Table 6. *Effect of nicotinamide on growth of Clostridium perfringens and ferrous sulphide formation in the presence of varying concentrations of sulphite in medium 2 in strains A and C*

Substance added:	—		Nic. amide		Nic. amide + various growth factors		—		Nic. amide	
Concn. of sulphite (μ mole/ml.)	Strain A		Strain A		Strain A		Strain C		Strain C	
	μ g. N/ml.	FeS	μ g. N/ml.	FeS	μ g. N/ml.	FeS	μ g. N/ml.	FeS	μ g. N/ml.	FeS
÷	39.9	÷	67.0	÷	51.9	÷	73.0	÷	78.0	÷
1	24.9	÷	67.1	(+)	51.9	(+)	77.0	÷	76.0	÷
2	24.0	(+)	61.3	++	51.9	+++	62.0	+	60	+
4	22.5	(+)	51.3	+++	47.1	+++	63.0	+++	61	+++
6	7.8	+	38.0	+++	18.4	+++
8	1.8	÷	39.0	+
10	0.0	÷	13.8	+	.	.	27.0	+++	61	+++
20	0.0	++	24.1	+++

Growth is expressed in μ g. N/ml.; ÷ denotes no ferrous sulphide formation, (+), +, ++, +++ denotes increasing degrees of blackening of the medium.

Peptone broth is rich in nicotinamide and apparently on subcultivation in this medium a 'negative' adaptation occurs, with resulting loss of ability to synthesize nicotinamide. The loss of this capacity did not affect the hydrogen sulphide formation from cystine in the medium, which continued unimpaired in the laboratory-maintained strains without adding nicotinamide to the defined medium 1. The hydrogen sulphide production from cyst(e)ine is thus independent of the reduction of sulphite; and the hydrogen sulphide formation from cystine and sulphite is additive, as estimated by the lead acetate and iron salt method. A black precipitate was not formed in medium which contained 3μ mole S/ml. as cystine, while the addition of 2μ mole S/ml. as sulphite resulted in the precipitation of ferrous sulphide both in the presence of 0.3μ mole and 3μ mole S/ml. as cystine. Therefore two distinct enzyme systems are concerned in the hydrogen sulphide production; this is also the case in *Proteus* (Tarr, 1934). The strains tested could not be 'trained' to dismiss one system with added nicotinamide by successive transfers in sulphite-containing medium 2, but their tolerance for sulphite was increased after subcultivation in sulphite-containing medium. Also the colony count in meat extract + peptone agar of the sulphite-adapted strain was much increased compared with the strain maintained on peptone water.

Growth experiments in the presence of atmospheric oxygen. It was shown by Quastel & Stephenson (1925) that certain facultative anaerobes grew anaerobically in the presence of nitrate in media which, in the absence of nitrate, could only support aerobic growth; the nitrate was reduced to nitrite. What is the significance of a corresponding reaction, the reduction of sulphite, in an anaerobic organism such as *Clostridium perfringens*? This organism was reported by Kligler & Guggenheim (1938) to grow aerobically in the presence of large amounts of ascorbic acid (0.02%). The addition of sulphite to our culture media did not allow 'aerobic' growth of *C. perfringens* in the medium 1 or 2.

Growth experiments in the presence of various carbon compounds and sulphite. In the defined medium *Clostridium perfringens*, like many anaerobes, can obtain the energy needed for growth only from carbohydrates (see Fuchs & Bonde, 1957). A series of experiments was therefore carried out to investigate whether *C. perfringens* could utilize other carbon compounds, which serve as substrates in aerobic life, as energy sources when sulphite was serving as hydrogen acceptor. A number of substrates which support aerobic growth in a variety of species, but not the anaerobic growth of *C. perfringens*, were tested in the presence of sulphite and nicotinamide and in absence of glucose. Of the substances tested the following did not support growth: formate, acetate, propionate, butyrate, succinate, succinate+fumarate, tartrate, citrate, pyruvate, lactate, gluconate, mannitol and ethanol. Slight growth occurred in glycerol, glycerophosphate and acetone.

Dehydrogenase activity in Clostridium perfringens

Different substances were investigated with methylene blue as hydrogen acceptor. Glucose, α -ketoglutarate, succinate, pyruvate, glycerophosphate and glycollate, and of amino acids, histidine and glutamic acid, were active in reducing methylene blue in the presence of washed suspensions of *Clostridium perfringens*. Lactate, propionate, butyrate, fumarate, malonate, arginine, phenylalanine, isoleucine, valine, aspartic acid, methionine and threonine were inactive. It was very difficult to obtain uniform suspensions, the rate of methylene blue reduction with the same substrate differed considerably in different preparations; therefore, reduction rates, to be comparable, should be obtained with the same suspension. Glucose was very rapidly dehydrogenated. Of the other substrates tested, only histidine reduced methylene blue at almost the same rate, α -ketoglutarate, succinate, glycerophosphate, glycollate, and glutamate were dehydrogenated by the suspensions at a much slower rate than glucose. Nevertheless, besides glucose, only glycerophosphate can support anaerobic growth and then very poorly. Of the amino acids reported by Stickland (1934) to serve as hydrogen donors in the reaction by which *C. sporogenes* obtains its energy, leucine, histidine and glutamic acid were also dehydrogenated by *C. perfringens* suspensions in the presence of methylene blue. Neutral red and Nile blue could also serve as hydrogen acceptors in these reactions.

Hydrogenase activity in Clostridium perfringens. In the first series of experiments methylene blue reduction in the absence and in the presence of gaseous hydrogen was compared, with and without added nicotinamide. The results obtained with three different strains are presented in Table 7. Clear acceleration of the reduction rate was observed in the presence of gaseous hydrogen, although the results were variable. Only with strain B was the reduction rapid, *C. perfringens* thus being able to activate molecular hydrogen.

The presence of a hydrogenase in a non-multiplying suspension of *C. perfringens* thus having been demonstrated, a second series of experiments was made to see whether sulphite could serve as hydrogen acceptor for molecular hydrogen.

Table 7. *Hydrogenase activity in Clostridium perfringens*

Reduction of methylene blue by non-multiplying suspensions of three strains of *C. perfringens* in the presence of molecular hydrogen.

Strain	Substrate	15 min.	30 min.	45 min.	60 min.	18 hr.	48 hr.
Degree % reduction methylene blue							
B	100 %	.
B	H ₂	80 %	95 %	95 %	95 %	100 %	.
C
C	H ₂	Some	Some	.	.	50 %	.
A	100 %
A	H ₂	100 %	.

B: newly isolated strain, grown in absence of nicotinamide. A and C: laboratory strains, grown in presence of nicotinamide.

Table 8. *Hydrogenase activity in Clostridium perfringens*

Reduction of sulphite by non-multiplying suspensions of two strains in the absence and presence of molecular hydrogen or glucose as hydrogen donors measured as blackening of lead acetate paper.

Strain	Substrate	Time of observation (hr.)				
		0.5	1	1.5	18	48
		Estimated degree of H ₂ S evolved				
A	Na ₂ SO ₃	—	—	—	—	+
A	Na ₂ SO ₃ + w.e.	—	—	+	.	+
A	Na ₂ SO ₃ + n.a.	—	—	+	.	++
A	Na ₂ SO ₃ + H ₂	+	+	+	.	++++
A	Na ₂ SO ₃ + H ₂ + w.e.	—	—	+	.	++++
A	Na ₂ SO ₃ + glucose	—	+	.	.	+++
A	Na ₂ SO ₃ + H ₂ + n.a.	—	—	—	.	—
A	Na ₂ SO ₃ + glucose + w.e.	—	—	—	.	+++
A	Na ₂ SO ₃ + glucose + n.a.	—	—	—	.	+++
B	Na ₂ SO ₃ + H ₂	—	—	—	++	.
B	Na ₂ SO ₃ + H ₂ + w.e.	+	.	.	++	.
B	Na ₂ SO ₃ + glucose	+	++	++	+++	.
B	Na ₂ SO ₃ + glucose + w.e.	+	.	.	+++	.
Boiled suspensions	Na ₂ SO ₃ + H ₂	—	—	—	—	—

w.e.: boiled water extract of the washed cells

n.a.: nicotinamide

The organisms were grown in the presence of nicotinamide and the rate of reduction was estimated by the degree of blackening of the lead acetate paper strip immersed in the centre well of the Warburg vessel used in the experiments. The results are presented in Table 8. As with methylene blue as hydrogen acceptor for hydrogenase, the results differed with different preparations, and the importance of extreme care and uniformity in the preparation of the bacterial suspension must be stressed.

In the presence of boiled organisms no hydrogen sulphide was produced from sulphite. The bacteria are able to utilize some internal compounds as hydrogen donors for sulphite reduction, although the reaction proceeds at a very slow rate in the absence of any added hydrogen donor. A water extract of washed organisms + nicotinamide accelerated this reaction, although it remains to be seen, whether they act really as carriers (or co-factors of another kind) or merely provide the organism with a hydrogen donor.

In the presence of molecular hydrogen, sulphite was reduced at much greater speed and much more hydrogen sulphide was produced (as judged by the degree of blackening of the lead acetate paper) than in its absence. When glucose was present, the hydrogen sulphide production was also rapid. From the preliminary experiments it may be concluded, that *Clostridium perfringens* possesses a hydrogenase system and is able to use molecular hydrogen for the reduction of sulphite, as well as other hydrogen-donors. Further experiments are planned to investigate the properties of these enzymes and to study quantitatively the reduction of sulphite to sulphide.

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Basic Triphenylmethane Dyes and the Inhibition of Glutamine Synthesis by *Staphylococcus aureus*

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SUMMARY: Glutamine synthesis by *Staphylococcus aureus* is inhibited by the basic triphenylmethane dyes crystal violet, methyl green, fuchsin, pararosaniline, brilliant green and malachite green. In the pH range 6.5-8.5, the inhibitory action of crystal violet is independent of pH, whilst with fuchsin, brilliant green and malachite green, the degree of inhibition is increased by increasing the hydrogen ion concentration of the medium. At pH 7.5, inhibitory activity increases in the order: brilliant green, fuchsin, malachite green, methyl green, crystal violet. The effectiveness of a basic triphenylmethane dye as an inhibitor of glutamine synthesis can be correlated with the basic dissociation constant of the dye.

During studies of the effects of various antibacterial agents on the uptake of glutamic acid by *Streptococcus faecalis* Gale & Mitchell (1947) found that certain basic triphenylmethane dyes, e.g. crystal violet, caused an increased accumulation of the amino acid inside the cells. Since the dyes did not affect the rate at which glutamic acid passed into the cells, the increased accumulation was apparently due to the inhibition of a metabolic pathway which normally decreases the cell's content of free glutamic acid. Crystal violet does not inhibit the deamination or transamination of this amino acid, nor its incorporation into peptides or proteins (Gale & Mitchell, 1947). During a search for enzyme systems which were inhibited by crystal violet, Elliott & Gale (1948) found that this dye inhibited the synthesis of glutamylhydroxamic acid by cell-free extracts of *Staphylococcus aureus*. Glutamylhydroxamic acid is formed when the enzyme preparation is incubated with hydroxylamine, L-glutamic acid, magnesium or manganese ions, and adenosinetriphosphate (Elliott & Gale, 1948). When the hydroxylamine is replaced by ammonia, the product of the reaction is glutamine (Fry, 1955). The work now described is a study of the effects of various triphenylmethane dyes on glutamine synthesis by cell-free extracts of *S. aureus* and an attempt to correlate their inhibitory properties with the basic dissociation constants of the dyes. A preliminary account of these experiments has been given previously (Fry, 1949).

The basic triphenylmethane dyes can exist in the form of coloured cations (as in the acid salts) or as undissociated bases, the carbinols (Fig. 1). The percentage of the dye in the cationic state is therefore dependent on the hydrogen-ion concentration of the system, and the greater the hydrogen-ion concentration, the greater the dissociation into the cationic form. Experiments with such dyes are complicated by two of their inherent properties. First, the

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equilibrium between the cation and the carbinol often requires an appreciable time to become established; and secondly, the carbinol form is relatively insoluble (Goldacre & Phillips, 1949).

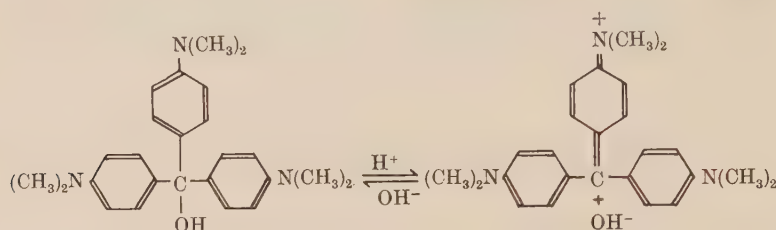


Fig. 1. The dissociation of crystal violet.

METHODS

Organism, growth medium, preparation of cell-free extracts. *Staphylococcus aureus* strain Duncan (Medical Research Council Unit for Chemical Microbiology, University of Cambridge) was grown at 37° for 6 hr. on a medium of initial pH 7.0 and containing 3% (v/v) tryptic or papain digest of casein (i.e. the equivalent of 3 g. digested casein/100 ml.), 0.1% (w/v) Marmite and 1% (w/v) glucose. The medium was dispensed in Roux bottles (150 ml./bottle). The organism was subcultured at 37° in test tubes containing 5 ml. of the same medium, and 0.5 ml. of a 16 hr. subculture was used to inoculate each Roux bottle. The organism was harvested by centrifuging, washed once with distilled water and then suspended in distilled water at a concentration equivalent to 30 mg. dry wt./ml. (for dry wt. determinations see Fry, 1955). The suspension was shaken with glass beads (Chance Bros. Ballotini no. 14) 10 g./tube, in a Mickle disintegrator (Mickle, 1948). After removing the beads by filtration, the filtrate was centrifuged at 1000 g for 30 min. The clear yellowish cell-free supernatant fluid was decanted from the debris and is the enzyme preparation used in all the reported experiments.

Chemicals. L-Glutamic acid (L. Light and Co. Ltd., Colnbrook, Bucks) was recrystallized as the hydrochloride. Adenosine triphosphate (ATP) was prepared from rabbit muscle (LePage, 1949) as the monobarium salt and solutions of the sodium salt were stored at -10° (Bailey, 1949). The glyoxaline buffers (Kirby & Neuberger, 1938) were prepared from the appropriate base hydrochlorides (British Drug Houses Ltd., London) and NaOH.

Dyes. Crystal violet was supplied by Hopkins and Williams Ltd., brilliant green, malachite green, fuchsin, methyl green, and pararosaniline by G. T. Gurr Ltd., and aurine by Imperial Chemical Industries. Crystal violet was recrystallized twice from hot water (Vogel, 1948); brilliant green and malachite green were purified by the methods of Lewis, Magel & Lipkin (1942). Dyes were added to the experimental systems in the form of an aqueous solution of their acid salts. The percentage of the dye in the cationic state was estimated colorimetrically (Goldacre & Phillips, 1949).

Analytical methods

Glutamine. Glutamine was estimated in terms of the ammonia liberated by hydrolysis with 5% (w/v) H_2SO_4 at 100° for 10 min. (Krebs, 1935). The procedure for the distillation of ammonia in the Parnas apparatus (Parnas & Heller, 1924) and its subsequent estimation by titration was the same as previously described (Fry, 1955).

Glutamylhydroxamic acid. Glutamylhydroxamic acid was determined colorimetrically by a method (Elliott, 1948) based on that developed by Lipmann & Tuttle (1945*a, b*) using synthetic glutamylhydroxamic acid as standard and a Hilger Spekker absorptiometer with a green filter (Ilford no. 604 with peak transmission at 520 m μ .).

Standard experimental systems

The standard experimental systems for studying the synthesis of glutamine consisted of 1 ml. enzyme preparation, 0.04M-4(5)-methylglyoxaline buffer pH 7.5, 0.01M- MgCl_2 , 0.1M sodium L-glutamate, 14.3 μ mole NH_4Cl and 0.006M-ATP in a total volume of 4.5 ml. Control systems did not contain added glutamate. Experimental systems were incubated in open test tubes in a water bath at 37° and the reaction was started by the addition of ATP after the tubes had been in the bath for 10 min. Where synthesis of glutamylhydroxamic acid was required, the NH_4Cl was replaced by 0.4M-hydroxylamine (Fry, 1955).

Results. Where applicable, results are expressed in terms of μ mole amide-nitrogen/total volume (4.5 ml.) of experimental system.

RESULTS

Inhibition of glutamine synthesis by crystal violet and methyl green

Elliott & Gale (1948) found that crystal violet at $2.5 \times 10^{-4}\text{M}$ inhibited the synthesis of glutamylhydroxamic acid. Preliminary experiments were therefore performed to determine the range of concentrations in which crystal violet exhibited any inhibitory action on glutamine synthesis. In these experiments, as in those of Elliott & Gale, the dye was added to the standard experimental systems a short time (less than 10 min.) before the enzyme preparation, and no account was taken of the fact that time might be required to establish an equilibrium between the cationic form and the carbinol of the dye. Concentrations of crystal violet greater than $6.2 \times 10^{-5}\text{M}$ produced progressive inhibition of glutamine synthesis (Fig. 2). Similar experiments were later performed with methyl green, and the inhibitory activity of this dye was found to be comparable with that of crystal violet (Fig. 2). Brilliant green, malachite green, pararosaniline and fuchsin were not as effective as crystal violet or methyl green. For example, whereas crystal violet at $1.2 \times 10^{-4}\text{M}$ produced 50% inhibition, concentrations of the order $7 \times 10^{-4}\text{M}$ of these four dyes were required to produce the same degree of inhibition.

Effect of pH value on the inhibitory activity of basic triphenylmethane dyes

The dyes selected for the study of inhibitory activity as a function of hydrogen-ion concentration were crystal violet, brilliant green, fuchsin and malachite green; the basic dissociation constants (pK) of these dyes have been determined by Goldacre, Phillips and other workers (Goldacre & Phillips, 1949). With dyes such as crystal violet, methyl green and fuchsin, the equilibrium between the cation and carbinol can be established in the standard

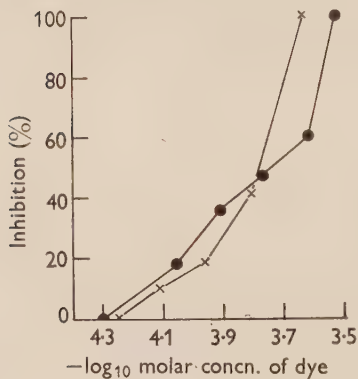


Fig. 2

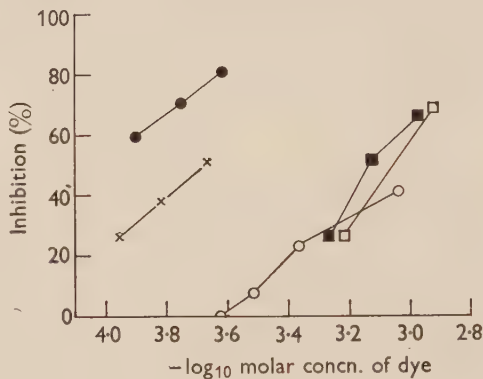


Fig. 3

Fig. 2. Inhibition of glutamine synthesis by crystal violet and methyl green. Complete and control systems: the standard experimental systems (see Methods, p. 343) with crystal violet (●) or methyl green (×) added to both as required. Incubated 1 hr. at 37°. Values in controls subtracted. Results compared with synthesis in the absence of dye and expressed as % inhibition. (Enzyme preparation in crystal violet experiments different from that used in methyl green experiments.)

Fig. 3. Inhibition of glutamine synthesis by crystal violet (●), methyl green (×), fuchsin (□), malachite green (■) and brilliant green (○). Complete and control systems: the standard experimental systems (see Methods, p. 343) with dye added to both as required 1 hr. before the addition of enzyme and ATP (8 hr. in the case of crystal violet). Incubated 1 hr. at 37°. Values in controls subtracted. Results compared with synthesis in the absence of dye and expressed as % inhibition. Same enzyme preparation used in all experiments.

experimental systems without precipitation of the carbinol. But the concentrations of brilliant green and malachite green which were required in these experiments were such that at certain hydrogen-ion concentrations the carbinol form began to precipitate. In these circumstances a true equilibrium could not be established.

The same enzyme preparation was used throughout the experiment and glutamine synthesis in different conditions of hydrogen-ion concentration was compared with that in the presence of known concentrations of the four dyes being studied. The dyes were incubated in duplicate experimental systems at 37° before the addition of the enzyme and ATP in order that conversion of the cationic form to the carbinol could take place. Samples from one set of experimental systems were used to estimate the amount of the dye in the

cationic state at the time when the enzyme preparation and ATP were added to the other set used to determine glutamine synthesis. The equilibration period allowed in the crystal violet experiment was 8 hr., since the equilibrium between cation and carbinol is established slowly (Goldacre & Phillips, 1949).

Table 1. *Effect of pH value on the inhibition of glutamine synthesis in Staphylococcus aureus by basic triphenylmethane dyes*

Complete and control systems: the standard experimental systems (see Methods, p. 343) with dye added to both as required. Incubated 1 hr. at 37°. % of dye as cation is the % of dye in the cationic state when enzyme preparation added. Dye incubated at 37° in experimental systems before addition of enzyme preparation: crystal violet, for 8 hr.; fuchsin, malachite green and brilliant green, for 1 hr. Different enzyme preparation for each dye.

Dye	pH	No dye			Dye added			Inhibition (%)	Proportion of dye as cation (%)		
		Control (A)	Complete system (B)	Activity (B-A)	Control (C)	Complete system (D)	Activity (D-C)				
			Amide nitrogen (μ mole)								
Crystal violet, 1.2×10^{-4} M	8.5	0.5	3.6	3.1	0.5	1.8	1.3	58	100		
	8.0	0.5	3.6	3.1	0.5	1.9	1.4	55	100		
	7.5	0.5	3.8	3.3	0.5	1.8	1.3	61	100		
	7.0	0.5	3.5	3.0	0.5	1.7	1.2	60	100		
	6.5	0.5	2.9	2.4	0.5	1.5	1.0	58	100		
Fuchsin, 1×10^{-3} M	8.0	0.3	4.5	4.2	0.3	3.8	3.5	17	10		
	7.5	0.3	4.6	4.3	0.3	3.9	3.6	16	10		
	7.0	0.3	4.2	3.9	0.3	2.7	2.4	39	24		
	6.5	0.3	3.1	2.8	0.3	0.9	0.6	79	52		
Malachite green, 7.6×10^{-4} M	8.0	0.3	3.5	3.2	0.3	2.8	2.5	22	8		
	7.5	0.3	3.5	3.2	0.3	2.6	2.3	28	8		
	7.0	0.3	3.0	2.7	0.3	1.6	1.3	52	26		
	6.5	0.3	2.6	2.3	0.3	1.1	0.8	65	36		
Brilliant green, 8.8×10^{-4} M	8.0	0.4	4.1	3.7	0.4	3.7	3.3	14	6		
	7.5	0.4	4.2	3.8	0.4	3.7	3.3	13	11		
	7.0	0.4	3.6	3.2	0.4	3.0	2.6	19	18		
	6.5	0.4	3.1	2.7	0.4	1.9	1.5	44	29		

In the pH range 6.5–8.5, the dye was 100% dissociated and the percentage inhibition due to 1.2×10^{-4} M crystal violet was not affected by change in pH value (Table 1). With fuchsin, the equilibrium was established within 1 hr., and with malachite green and brilliant green, because of precipitation of the carbinol, the 'equilibration' period was arbitrarily fixed at 1 hr. An increase in the hydrogen-ion concentration of the system was accompanied by an increase in the percentage inhibition of glutamine synthesis by fuchsin, malachite green and brilliant green (Table 1). The amounts of these three dyes in the cationic form increased with increase in the hydrogen-ion concentration of the system, and this result was not unexpected since the pK values for fuchsin, malachite green and brilliant green are *c.* 7.0, 6.9 and 7.9, respectively (Goldacre & Phillips, 1949). There would thus appear to be a correlation between percentage inhibition and the degree of dissociation of the dye. If this were true, then the order of effectiveness of the dyes at a given pH value should be

related to the values of their basic dissociation constants. The effect of five dyes at pH 7.5 was determined and when activity is expressed in terms of the concentration producing 50 % inhibition, then the following order of increasing effectiveness was found: brilliant green, fuchsin, malachite green, methyl green, crystal violet (Fig. 3). The dyes fell into two groups: crystal violet and methyl green were the most active inhibitors of glutamic synthesis, and malachite green, fuchsin and brilliant green were about half as effective. The pK of crystal violet is 9.3 and though the pK of methyl green has not been determined, it is probably of the same order because of the quaternary nitrogen structure of the dye (see Kligler, 1918; Gale & Mitchell, 1947). The pK values of the other three dyes are in the range 7–8, consequently at pH 7.5, crystal violet and methyl green are mostly, if not completely, dissociated, whereas the other three dyes are about 50 % or less dissociated into the cationic form. It is therefore concluded that the inhibition of glutamine synthesis by the basic triphenylmethane dyes is due to the cations of the dyes and that for each dye the degree of activity is related to the dissociation constant of the equilibrium between the carbinol and cation of the dye.

The dyes used in all the above experiments were basic and for comparison, the effect of an acidic triphenylmethane dye, aurine, was determined. The synthesis of glutamylhydroxamic acid by cell-free extracts of *Staphylococcus aureus* was not affected by concentrations of aurine up to 5×10^{-4} M (this is twenty times the concentration of crystal violet which produced complete inhibition with the same enzyme preparation). Though aurine inhibits the internal metabolism of glutamic acid in *Streptococcus faecalis*, this result supports the view of Gale & Mitchell (1947) that the mode of action of aurine is different from that of the basic triphenylmethane dyes.

DISCUSSION

The results reported here lead to the conclusion that it is in the form of their cations that the basic triphenylmethane dyes inhibit glutamine synthesis. The inhibitory effects are therefore presumably due to combination of the dye cations with negatively-charged groups in the enzyme system, either those present in the enzyme proteins or perhaps in a co-factor. The importance of thiol groups in the synthesis of glutamine in *Staphylococcus aureus* has already been established (Fry, 1955) though whether or not they are protein in nature, or present in a coenzyme such as coenzyme A, has not been established. In the range of hydrogen-ion concentration used in these experiments, the thiol groups would be negatively charged; it is therefore possible that such groups would attract the positively charged ions of the dye and thus form an addition complex which is not easily dissociated. In such circumstances, it is to be expected that the activity of the enzyme would be decreased. Alternatively, the dye cations may be attracted to any negatively-charged groups in the enzymic system and by steric hindrance make it impossible for the substrates to reach the appropriate receptor areas on the enzyme. Another way in which the dyes might exert their effects is by occupying the position of the

normal cationic activator of the system, i.e. by replacing magnesium or manganese ions. Such an explanation is feasible because staining of bacteria by basic triphenylmethane dyes leads to the displacement of cations such as magnesium ions or hydrogen ions from the cells (McCalla, 1941).

From Table 1 it can be seen that the basic triphenylmethane dyes become more effective as the hydrogen-ion concentration of the system is increased, and in consequence the dyes become more dissociated into cations. Such results are to be compared with those of Quastel & Yates (1936) concerning the inhibition of saccharase by various basic dyes. They found that the degree of inhibition increased as the hydrogen-ion concentration of the medium decreased. It was suggested that the higher the pH value, the greater the binding of the dye cations to the negatively charged enzyme protein. The dyes were fully ionized in the experimental conditions employed (pH range 3.5–6.0) and consequently there were no complications concerning the influence of pH value on the dissociation of the dye.

Arising out of their studies of the staining of bacteria by basic and acidic dyes at various pH values (Stearn & Stearn, 1924*a*), Stearn & Stearn (1924*b*) were the first to suggest that the bacteriostatic powers of the basic triphenylmethane dyes were due to the coloured dye cations combining with the acidic groups in the bacterial protein, and that the greater the basicity of the dye, the greater its expected antibacterial action at physiological pH values. The Stearns, however, also realized that factors other than basicity may influence the absorption of dyes by intact bacteria. Kligler (1918) had already shown that the greater the number of substituent alkyl radicals in the amino groups of the triphenylmethane dyes, the greater the antibacterial activity. An exception was methyl green which, although containing seven methyl groups in the molecule, was virtually inactive. The activity of the basic triphenylmethane dyes in preventing the growth of *Streptococcus faecalis* and as inhibitors of the internal metabolism of glutamic acid in washed suspensions of the organism was later correlated with the apparent lipid/water partition coefficient of the dyes (Gale & Mitchell, 1947). This work indicated that the antibacterial properties of these dyes is a reflexion of the ease with which the dyes penetrated the cell walls of the bacteria. Lipid solubility will be influenced by the number and type of the alkyl groups in the dye molecule and since the bacterial plasma-membrane is believed to be composed of lipoprotein (see Mitchell & Moyle, 1956), it is reasonable to suggest that the greater the lipid solubility of the dye, the greater its antibacterial activity. The apparent partition coefficient between *isobutanol* and water for methyl green is very low, and Gale & Mitchell (1947) therefore concluded that the feeble antibacterial activity of the dye was due to its inability to enter the cell. However, once inside the cell, antibacterial powers of the dyes probably lie in their ability to inhibit one or more essential enzyme systems. The results given in the present paper indicate that as far as glutamine synthesis is concerned, once the basic triphenylmethane dyes have penetrated to the site of action, their effectiveness is related not to their lipid solubility but to their basic dissociation constants. A summary of the known inhibitory effects of the triphenylmethane dyes is

given in Table 2, in the last three columns of which the figures refer to the dyes arranged in order of increasing activity.

Acetylhydroxamic acid is a compound analogous to glutamylhydroxamic acid and in *Escherichia coli* the synthesis of the former compound involves the formation of an energy-rich compound, acetyl phosphate, from acetate and

Table 2. *Summary of certain properties of basic triphenylmethane dyes*

Sources of data: (a) Goldacre, Phillips & Rumf (Goldacre & Phillips, 1949); (b), (c) and (d), Gale & Mitchell (1947) for *Streptococcus faecalis*; (e) present paper, synthesis of glutamine by cell-free extracts of *Staphylococcus aureus*. In columns (c), (d) and (e) numbers refer to position of dyes when placed in order of increasing activity, the larger numbers referring to the higher activities.

Dye	Substituent groups	pK (a)	Partition coefficient butanol/water (b)	Degree of inhibition of growth (c)	Degree of inhibition of glutamate metabolism; intact organisms (d)	Degree of inhibition of glutamine synthesis; cell-free extracts (e)
Methyl green	-N(CH ₃) ₃ Cl -N(CH ₃) ₂ -N(CH ₃) ₂	—	0.2	1	1	4
Fuchsin	-NH ₂ -NH ₂ -NH ₂ -CH ₃	c. 7.0	14.7	2	2	2
Crystal violet	-N(CH ₃) ₂ -N(CH ₃) ₂ -N(CH ₃) ₂	9.4	76	3	3	5
Malachite green	-N(CH ₃) ₂ -N(CH ₃) ₂	6.9	125	4	4	3
Brilliant green	-N(C ₂ H ₅) ₂ -N(C ₂ H ₅) ₂	7.9	480	5	5	1

ATP and then a spontaneous reaction between the acetyl phosphate and hydroxylamine (Lipmann & Tuttle, 1945*a, b*). Though the experimental conditions for the synthesis of glutamylhydroxamic acid are similar, an analogous reaction mechanism does not appear to operate since, although crystal violet inhibited the synthesis of glutamylhydroxamic acid, it had no effect on the synthesis of acetylhydroxamic acid (B. A. Fry, unpublished). This supports previous conclusions, since all attempts to show that glutamyl phosphate is an intermediate in glutamine or glutamylhydroxamic acid synthesis have so far failed (Elliott, 1951; Speck, 1949; Fry, 1955).

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Immunological Studies of the Immobilization Antigens of *Paramecium aurelia* variety 2

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SUMMARY: An immunological study was made of *Paramecium aurelia* variety 2. The antigens which produce immobilization antibodies were studied and a survey of the antigenic types, or serotypes, manifested by variety 2 stocks carried out. The eight types found were titrated against several sera. Serotype B₂ when found in different stocks, was distinguished from stock to stock on the basis of reactions to homologous and heterologous sera. Stock-specific C serotypes were also found and the conditions for maximum stability of the C serotype occurring in different stocks determined. It has been shown by absorption experiments that: (a) antiserum against a single serotype has at least two antibodies directed against the immobilization antigen(s); (b) if there are two or more determinant groups in a serotype, these groups are on a single molecule. The possible serological bases for cross-reactions of serotypes controlled by the same genetic locus are discussed.

The functions of the nucleus and cytoplasm in heredity and differentiation have been investigated extensively in *Paramecium*. Studies by Sonneborn & LeSuer (1948) and by Sonneborn (1948, 1950*a*) with varieties 4 and 8 of *Paramecium aurelia*, and by Beale (1952) with variety 1, were concerned specifically with the inheritance of immobilizing antigens. The work reported here deals with immunological studies of similar antigens found in variety 2.

Cultures of *Paramecium aurelia* when injected into rabbits elicit antibody formation against paramecia of the injected type. Serum prepared against clones of paramecia pure for a specific antigenic type will, in proper concentrations, immobilize those organisms which have the same immobilizing antigens as the paramecia injected and will not react with other paramecia. When such resistant forms are in turn used to produce antiserum, the new serum will leave unaffected those paramecia which are of the type used for the first injection. Using this procedure, Sonneborn & LeSuer (1948) demonstrated that a single stock can give rise to several different antigenic types or serotypes which can be told apart on the basis of their reaction to various sera.

As an essential preliminary to immunological and genetic studies of the serotypes a survey of the antigenic types manifested by variety 2 stocks has been made and the conditions for the stability of certain serotypes determined. Absorption experiments have also been performed in an attempt to determine the basis for cross-reactions of serotypes controlled by the same genetic locus found in different stocks. It has been shown that the immobilization determinant groups found in a specific serotype are located on a single molecule.

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METHODS

Organisms. The following stocks of *Paramecium aurelia* variety 2, collected in the United States, were used: 1 from Strickersville, New York; 7, Pinehurst, North Carolina; 21, Woodstock, Maryland; 30, Coates Pond, Maryland; 35, Moscow, Indiana; 53, Twin Lakes, Indiana; 72, Pyote, Texas; 83, Millbrook, New Jersey; 85, Blairstown, New Jersey. A single derived stock, d30-2, was also used; these organisms were obtained by crossing stocks 7 and 30 and inducing in the hybrid offspring a nuclear reorganization, autogamy, which ensured homozygosity at all loci. These exautogamous paramecia were capable of manifesting serotype 30-C.

Cultivation. Stocks and all clones derived from them were kept at temperatures from 12° to 31°, as the experiment required, in a baked lettuce medium or, less frequently in a 0.075 % Cerophyl infusion (Cerophyl tablets or powder obtained from Cerophyl Laboratories, Kansas City, Kansas, U.S.A.) inoculated with *Aerobacter cloacae*. Cerophyl medium was prepared by dissolving 11.3 g. of Cerophyl in 1 l. of distilled water, boiling slowly for 3-4 min. and then filtering through cotton. The filtrate was added, with 17 g. Na_2HPO_4 , to 15 l. distilled water and autoclaved at 15 lb./sq.in. for 30 min. Sonneborn's review of methods in the biology of *Paramecium* should be consulted for details of cultivation and for other methods mentioned below (Sonneborn, 1950*b*).

Serological methods. Most of the immunological techniques used in these studies were described in detail by Sonneborn (1950*b*) or are described below in specific sections of the results.

Absorption experiments were carried out with paramecia grown in 2 l. flasks or in 15 l. of culture fluid in large jugs. Whole organisms, breis, soluble antigens or lyophilized antigens were added to inactivated (56° for 30 min.) serum and incubated for 90 min. at 27°. The antigen serum mixture was then centrifuged for 2 min. in a clinical centrifuge at 720 *g* and the supernatant fluid titrated for immobilization activity. The paramecia were prepared in the four ways mentioned for these experiments as follows:

(1) *Whole organisms* were filtered through sixteen layers of cheese cloth before being centrifuged for 2 min. in an International electric oil-testing centrifuge at 450 *g* or in a small clinical centrifuge until the desired concentration was reached.

(2) *Breis* were prepared by concentrating the organisms as above, or by using a modified cream separator which was adjusted to allow a rotor speed of 3450 rev./min. (700 *g*; Dr J. R. Preer, private communication). A Farm Master electric cream separator, 600 pounds capacity (Sears, Roebuck and Co., Philadelphia, Pennsylvania, U.S.A.), was fitted with a pulley of 3 in. diameter to replace the original 5 in. pulley attached to the motor shaft. An endless belt connected this substitute pulley with the spindle of the rotor so that for every turn of this small pulley the rotor revolved about three times. The cream opening in the rotor was completely closed.

The concentrated suspension of paramecia was emptied from the rotor after 15 l. or less had been centrifuged and was placed in a large beaker or flask so as

to have a high proportion of the 300 ml. of concentrate exposed to air. When the desired concentration was reached, following further centrifugation in an oil-testing centrifuge, the paramecia were homogenized in the cold. The brei was either incubated immediately with serum or kept in a deep freezer until needed.

(3) *Soluble antigen* was extracted simply by centrifuging either a freshly prepared brei or the stored brei in a refrigerated centrifuge at 24,000 g for 1 hr. at 0° and using the decanted supernatant fluid as antigen.

(4) *Lyophilized antigen* was prepared by concentrating whole organisms to c. 15 % pure paramecia by volume. The final volume, not exceeding 15 ml., was frozen immediately at -70° and placed under vacuum in a glass lyophilizing apparatus for 4-10 hr. The lyophilized antigen was stored with a desiccant in a refrigerator.

RESULTS

Survey of stocks

A survey of the antigenic types which variety 2 stocks can manifest has been carried out by Dr J. R. Preer (private communication) using the methods of Sonneborn (1950*b*). The general procedure followed was first to obtain antisera by injecting samples of clones into rabbits. Then samples of a number of clones from the same stock as that of the injected paramecia and from other stocks were exposed to these antisera. Paramecia immobilized were classified as having the same serotype as the paramecia against which the sera were originally prepared. Unaffected paramecia were cultivated separately and samples subsequently injected and the new serum collected. This new serum immobilized paramecia which bore the same immobilization antigens as did the injected paramecia.

Eight serotypes were found in variety 2 stocks: A, B, C, D, E, F, G and H. All of these serotypes were tested with sera prepared against serotypes found in varieties 4 and 8 by Sonneborn. Paramecia which were immobilized in one of the specific antisera were classified as being of that specific serotype which was used in the preparation of the antiserum. In this way variety 2 paramecia were designated as serotypes A, B and G because they were immobilized by Sonneborn's specific anti-A, anti-B and anti-G sera, respectively. Dr J. R. Preer discovered that the five other serotypes were unaffected by Sonneborn's antisera. These serotypes were therefore given different letter designations from those serotypes of varieties 4 and 8. Table 1 lists the serotypes and the titres, the most dilute concentration in which immobilization occurs, against eight sera.

Cross-reactions between serotypes found in different stocks

The same serotypes found in different stocks generally cannot be told apart even when several homologous and heterologous sera are used for identification. Thus, paramecia of serotype G, a serotype which has been found in every stock of variety 2 which has been tested, have identical titres with a number of anti-G sera. Serotype E of several stocks has shown a similar uniformity of

reactions. On the other hand, in variety 1 Beale found genetically homologous serotypes, i.e. serotypes whose specificity is controlled by the same genetic locus, which exhibit almost no immunological cross-reactions (Beale, 1952).

Table 1. *Titration of sera prepared against eight serotypes of Paramecium aurelia variety 2*

Serum	Prepared against		Stock and serotype exposed to sera							
	Stock	Type	53-A	53 × 30-B	30-C	30-D	53-E	7.s-F	30-G	5-H
10	53	A	7, 9	3, 5	—, —	—, —	—, —	—, —	—, —	—, —
12	53 × 30 hybrid	B	1, 3	7, 9	—, 2	. .	—, 3	2, 5	6, 8	—, —
8	30	C	—, 2	. .	7, 8	—, —	1, 2	—, —	—, 4	—, —
6	30	D	—, —	. .	—, —	7, 8	—, —	—, —	—, —	—, —
3	53	E	—, 1	. .	—, —	—, —	7, 9	—, —	—, 1	—, —
11	7.s	F	—, 1	—, —	—, —	6, 8	—, —	—, —
7	30	G	3, 5	—, —	—, —	—, —	2, 3	—, —	7, 10	—, —
15	5	H	5, 7

The three columns on the left refer to sera. The figures in the body of the table represent the lowest concentration of the serum that will immobilize the animals in 2 hr. at 27° (the first figure given), and the lowest concentration that will retard the animals (the second figure). The figures represent concentrations of serum as follows:

$\frac{\text{Ml. diluent + serum}}{\text{ml. serum}}$	12.5	25	50	100	200	400	800	1600	3200	6400
Dilution number	1	2	3	4	5	6	7	8	9	10

(—) means no reaction; (·) means not tested.

In the variety 2 stocks, 1, 30, 50, 72 and 83 in which B serotypes are found, four main types of B can be distinguished on the basis of the similarities and differences of their reactions with two sera. 83-B and 1-B represent one type, 72-B, 50-B and 30-B three more types (Table 2).

Table 2. *Titration of serotypes B and C found in different stocks of Paramecium aurelia variety 2*

Serum	Prepared against		Serotypes tested				
	Stock	Serotype	72-B	30-B	50-B	83-B	1-B
F#6	72	B	4, 8	—, 2	3, 4	—, —	—, 3
P#12	53 × 30 hybrid	B	5, 8	7, 9	3, 5	3, 5	3, 5
Serum	Stock	Serotype	Serotypes tested				
			30-C	85-C	7-C	21-C	
F#3	d30-2	C	6, 8	5, 7	4, 7	4, 5	
P#9	7	C	5, 8	4, 6	6, 9	2, 4	

For legend see Table 1.

Serotype C also differed in various stocks (Table 2). Thus, by the combined use of two sera (F#3 and P#9) it was possible to differentiate 7-C, 21-C and 30-C. The C in stock 85 appeared to be virtually identical with that in stock 30.

Stability of serotype C

Serotype C was found to be rather unstable in stock 30. However, in the derived line d30-2 (a homozygous line containing genes from stock 7) it was quite stable. Consequently, the paramecia manifesting the 30-C serotype in all the experiments reported below were taken from d30-2. In these lines 30-C was quite stable at 17°, 27° and 31°, with 17° the most favourable. Rate of feeding had no effect on stability. The difference in stability of serotype 30-C in stock 30 and line d30-2 suggests that genes from stock 7 play an important part in determining the stability of this serotype (see Sonneborn, Ogasawara & Balbinder, 1953). Indeed serotype C is considerably more stable in stock 7 than in stock 30. It is most stable, however, in d30-2. In stock 7 it is quite stable at 12° and 17° but transforms to another serotype at 27° and higher. To provide an environment in which both serotypes 30-C and 7-C had approximately equal opportunity for expression, clones were placed at 17° with enough culture fluid to permit fission rate of 0.5, 1.0, or 1.5 fissions/day.

Immunological basis for cross-reactions

The relationship of immobilizing antigens of the C serotype to homologous and heterologous sera was studied to determine the basis for cross-reactions of serotypes controlled by a single genetic locus. Two hypotheses will be considered as possible explanations for the observed cross-reactions. One assumes quantitative differences in the antigens, the other assumes qualitative differences. According to either hypothesis, however, a serum prepared against one antigen possesses more than one kind of antibody that will react with this antigen.

A series of absorption experiments was performed to determine whether serum prepared against one serotype has more than one kind of immobilizing antibody against this serotype. When antigens, either in the form of whole organisms, breis, soluble antigen or lyophilized antigen, were added to homologous antiserum and the serum titrated for the ability to immobilize 7-C and 30-C organisms, both heterologous and homologous titres were greatly decreased or completely removed. When the proper concentrations of paramecia of the heterologous serotype were chosen, it was possible to absorb out completely all heterologous activity (five dilutions, i.e. from 1:200 to 0) from this same antiserum without lowering the homologous titre more than two dilutions (Fig. 1). If only one type of antibody were present, lowering the titre against one serotype by two dilutions should lower the titre against all cross-reacting serotypes a like amount. Therefore, at least two kinds of immobilizing antibodies must be elicited by the injection of organisms of a single serotype.

Now, according to the first hypothesis, a multiplicity of determinant groups may be present in a serotype, and although all may be present in each serotype, their ratio may vary in the two. If separate antibodies were formed against the various groups, then cross-reactions would be obtained. Absorption of antiserum by a sufficient quantity of heterologous antigen should completely

remove all antibodies. The first hypothesis, then, requires the demonstration of the presence of the same immobilizing antigens in cross-reacting serotypes. According to this hypothesis large numbers of 7-C paramecia when added to 30-C antiserum should be able to remove completely all immobilization anti-

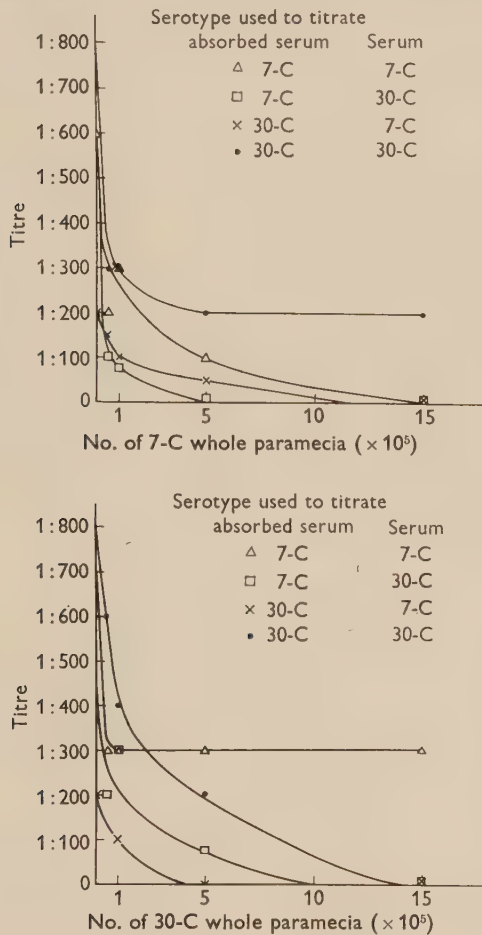


Fig. 1. Absorption of anti-C serum with different kinds of serotype absorbents. The absorbent concentration is given in terms of numbers of paramecia/ml. serum. The curves represent the change in immobilization titre of a serum against a particular C serotype after serum and absorbent had been incubated. Absorption with 7-C antigen preparations gave comparable curves, including 7-C soluble antigen.

body against 30-C paramecia. But even when 20,000,000 lyophilized paramecia/ml. serum were added to 30-C antiserum, the quantity of 30-C antibodies was not lowered below dilution 4 or 5, a level first reached with as few as 50,000 paramecia/ml. serum. The existence of a plateau which cannot be lowered indicates that there is at least one antibody present in homologous serum with which heterologous organisms do not react. The proportion of all the immobilizing antibodies in the serum that can combine with homologous

organisms only determines where this plateau levels off for a particular serum. Thus, a serum whose homologous titre can only be decreased from dilution 6 to 4 by a cross-reacting serotype has 25 % of its content antibody unable to react with heterologous organisms (100 (dilution 4):400 (dilution 6)). This 25 % therefore represents that antibody which combines with antigen present in only one of the serotypes, the homologous type. It may, therefore, be concluded that the first hypothesis, requiring the presence of different amounts of the same antigens in cross-reacting serotypes cannot, by itself, account for cross-reactivity.

It appears, then, by elimination of the first hypothesis as likely to account for the cross-reactions of serotypes, that an alternative explanation of a qualitative difference between serotypes is more reasonable. For instance, a multiplicity of determinant groups may be present, some common to both cross-reacting antigens, while others are specifically located in only one. If antibody were formed against common groups, it would react with both antigens. Antibody formed against the specific groups would react with only one of the antigens. That antibodies with such specificities are indeed formed in the case of azo-substituted proteins has been shown by Heidelberger & Kendall (1934); absorption of antiserum by heterologous antigen should remove only the common antibody leaving behind the specific antibody. Hooker & Boyd (1934) interpreted their results with hen and duck albumin according to this hypothesis.

Location of immobilization determinant groups

If at least two immobilization combining groups are responsible for the specificity of a serotype, one cross-reactive group and one group not reacting with other serotypes controlled by the same locus, it would be of interest to know whether these combining groups for specific reactions and for cross-reactions are on a single molecule. To determine the locations of these groups the following experiment was performed.

A soluble antigen was extracted from 7-C paramecia and added to 30-C antiserum so that almost all 7-C antibodies would be removed while, at the same time, the 30-C titre would remain high. To the absorbed supernatant fluid was added 7-C antiserum which had previously been absorbed with enough extract of 30-C paramecia to remove almost all the 30-C antibodies, as determined by immobilization tests, and to leave almost unaltered the 7-C titre. The antigen concentration for both 7-C and 30-C paramecia was 200,000 paramecia/ml. undiluted serum. After incubation the mixed sera were centrifuged and the supernatant fluid titrated with the results shown in Table 3.

If the immobilization antigens had been on separate molecules, the 30-C antiserum absorbed with 7-C antigen should have in its supernatant 7-C specific antigen since this antigen would not be brought down with the fraction that would remove the cross-reacting antibodies. Similarly, the absorbed 7-C antiserum would also contain 30-C specific antigen. When the two absorbed sera are mixed the titre of this mixed serum against both 7-C and 30-C paramecia should be lowered. Instead, the serum behaved as though its two component

sera had no antigen molecules present: the titre of the mixed absorbed sera remained high against both serotypes. The alternative expectations for this experiment are shown in Fig. 2. Apparently, then, if there are two immobilization combining groups in a serotype, they behave as a unit. When one combining group is bound to an antibody, as in absorption, the other immobilization-combining groups are also tied up even though not reacting with the antibody.

Table 3. *Titration of supernatant mixtures of absorbed antisera for presence of free antibody*

The titres are expressed as the reciprocals of the dilutions of the sera.

	Titre against 30-C paramecia	Titre against 7-C paramecia
Unabsorbed 30-C antiserum	200-400	50-100
Unabsorbed 7-C antiserum	25-50	100-200
30-C antiserum absorbed with 7-C paramecia	50-100	12-5
7-C antiserum absorbed with 30-C paramecia	12-5	50
Mixture of the supernatant fluids of the two absorbed sera	100-200	50-100

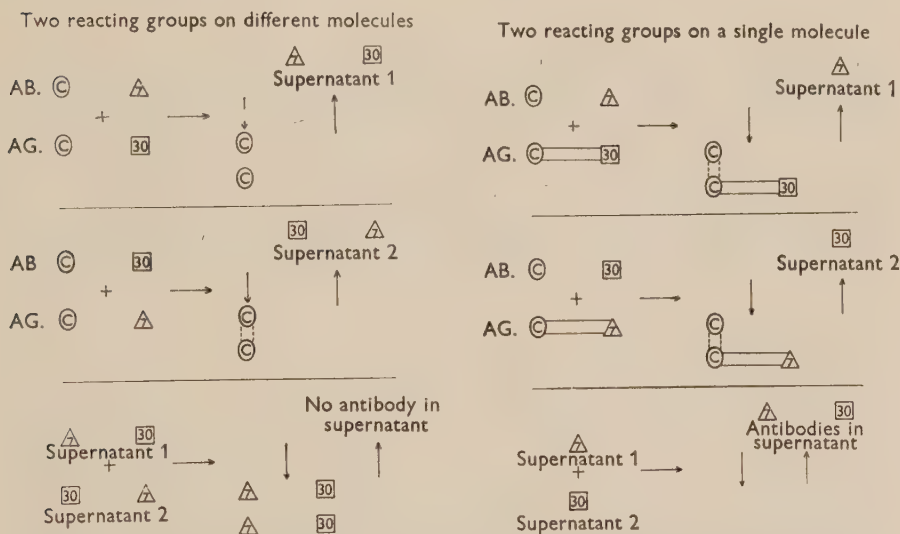


Fig. 2. Absorption experiment to determine if determinant groups are on separate molecules. AB. = antibody. AG. = antigen.

DISCUSSION

Although only one qualitative difference between antigens has been suggested above (that involving common and specific determinant groups as explaining cross-reactions) there is another possible qualitative mechanism which does not assume separate antibodies against distinct determinant groups. The two antigens might be closely related but show small chemical or structural differences. Antibodies would be directed against the antigen molecule as a

whole, but the various antibody molecules would not be homogeneous, differing in their affinity for the homologous antigen. A mechanism such as this was suggested by Landsteiner & van der Scheer (1936) as the most reasonable explanation for some of their results from using artificial conjugated azo-proteins. The basis for cross-reactivity according to this view would reside in the lack of uniform reactivity of the antibody molecules formed against the homologous antigen, for the heterologous and homologous antigens would not be expected to show the same affinities for the different antibody molecules.

The basis for cross-reactivity of a serotype with sera prepared against other serotypes within the same stock (as contrasted with cross-reactions between the same serotype in different stocks) is still unknown. An analysis similar to the one carried out on the stock-specific C serotypes using absorption techniques would be expected to be of similar value in discovering the cause of this kind of cross-reactivity. However, even if an antigen common to serotypes from different stocks were the cause of the cross-reactions shown by these stock-specific serotypes, it would seem less likely that a common antigen would account for the cross-reactions of serotypes determined by different loci. Alleles at a locus might be expected to control the synthesis of similar products, but this would appear to be less likely in the case of genes situated at entirely different loci.

Sonneborn (1947), on the basis of early absorption studies, suggested that there exist in paramecia of a particular serotype, two classes of antigens. One of these classes, the secondary antigens, does not take part in the immobilization reaction, but can induce the formation of antibodies capable of immobilizing a second serotype. This second serotype possesses this secondary antigen as a primary antigen, one capable of reacting with immobilization antibodies. Such secondary antigens should be capable of detection by absorption experiments. Later more extensive studies carried out with sera possessing high titres of immobilizing antibodies completely failed to reveal secondary antigens, e.g. by comparing absorption with whole paramecia and with breis (Dr T. M. Sonneborn, personal communication).

Experiments reported elsewhere with variety 2 (Finger, to be published), 4 (Sonneborn, 1950*a*) and 1 (Beale, 1952), have demonstrated that serologically related serotypes found in different stocks are determined by alleles. It is not known whether the converse is true, that unrelated serotypes are determined by allelic genes in variety 2, because the necessary crosses have not been made. Beale's studies with variety 1 (1952) included crosses between both serologically related and serologically unrelated serotypes, and in both instances he found that a single locus might control cross-reactive serotypes and non-cross-reactive serotypes. In other words, it is possible to have genetic homology without a corresponding serological homology.

A plausible explanation for the fact that a locus may control serotypes which have almost no serological relationship, as determined by the immobilization test, is that this test may be extremely sensitive. Thus, although serotypes which may have almost identical antigenic configurations cannot be told apart, as may be the case with the G serotypes, serotypes which differ only slightly.

such as perhaps the various B and C serotypes, may be distinguished with little difficulty. On the other hand, should two serotypes resemble each other only slightly, on the basis of their immobilization reactions they may be classed as distinctly unrelated types.

It may be concluded that a further study of the immunological relationships of cross-reacting serotypes may still be considered a fruitful approach to the problem of gene action. Similarly, the general problem of the differentiation of cells with like genomes may be approached through an immunological investigation of the relation of serotypes controlled by different genetic loci. Although this relationship may not be demonstrated by means of the immobilization test, it is possible that the application of other immunological techniques, such as gel diffusion methods (Preer, to be published; Finger, to be published), may prove valuable in detecting such relationships, if they do indeed exist.

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Relationships between Cultural Characters and Pathogenicity in *Venturia inaequalis* and *Venturia pirina*

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SUMMARY: Series of experiments were carried out both *in vitro* and *in vivo* on single conidial isolates of *Venturia inaequalis* and *V. pirina*. Relationships between colony type and sporulation *in vitro* were apparent in both species. In addition, isolates of *V. inaequalis* showed relationships between degree of resistance of host source, width of host range and stability of cultural characters and pathogenicity during storage in culture. Isolates of *V. pirina* showed no such relationships, cultural characters being invariably stable while pathogenicity rapidly declined in the storage conditions. Studies of nutritional requirements suggested no deficiencies in the synthetic abilities of either pathogen, but limiting nutritional factors were important. Isolates of both species which differed widely in pathogenicity and other characteristics, reacted similarly to various culture media.

The use of *Venturia inaequalis* (Cke.) Wint. as a test organism for the laboratory evaluation of protective fungicides for use against the apple and pear scab diseases (Montgomery & Moore, 1938) requires the provision of a routine supply of conidia over long periods. Much difficulty has been experienced in maintaining this supply without frequently obtaining new isolates, and the present study was therefore initiated to investigate sporulation in artificial culture and to determine optimal methods and media. Modifications in the method of fungicide test have been reported (Kirby & Frick, 1952) and in these, some results of this study have been applied. The scope of the work was later widened to include *V. pirina* Aderh. and studies of possible relationships between cultural characters and pathogenicity in the two species.

METHODS

Conidia were isolated from fruit or leaf lesions. They were allowed to germinate on 2% (w/v) malt-extract agar at 18° and then isolated singly on small blocks of agar, using a platinum-iridium needle. The blocks were transferred to 2% malt-extract agar slopes and, after 14 days' incubation at 18°, the cultures were stored at 5° under sterile B.P. grade paraffin oil to a maximum depth of 1 cm. above the upper edges of the colonies (Buell & Weston, 1947). Ascospores were collected from moistened overwintered leaves and isolated in the same way.

The morphological differentiation of the isolates was carried out on a standard depth of 2% (w/v) malt-extract agar, at pH 5.6 before sterilization. The growth of four replicate conidia of each isolate was described and recorded photographically after 6 weeks of incubation at 18°; 2% malt-

extract was chosen, as maximum differentiation occurred most rapidly on this medium.

Comparative sporulation of the isolates was determined turbidimetrically after growth on filter-paper cylinders standing in nutrient solutions (Kirkham, 1956). In all tests each treatment was applied in duplicate and the turbidity estimate expressed as the geometric mean of a pair of observations. Geometric means were used so that variability of the means could be expressed in terms of a significant ratio, since it was found that the standard deviation of an observation was proportional to its mean value. Two means differed significantly (5% level of significance) when their ratio exceeded 1.38. Growth on the paper cylinders was recorded at the cultural endpoints, using the following categories: 0 = none or traces; 1 = thin or inhibited; 2 = normal but suboptimal; 3 = optimal.

The pathogenicity and host ranges of isolates were determined with apple and pear trees, potted in John Innes Compost No. 1, in their second year of growth. Groups of trees were pruned to provide a succession of growing extension shoots during the season. Each scion was initially cut back to 3 ft. above the graft union, and a maximum of four buds in the apical zone allowed to produce shoots. The potted trees were plunged in the open and moved batchwise into the greenhouse a month before they were required for inoculation, to avoid the risk of natural infection by *Venturia* spp.

Shoots between 7 and 12 in. in length were used. The first fully expanded leaf below the shoot tip was labelled on the petiole and designated L₀, to indicate the stage of development at the time of inoculation. Each shoot was enclosed in a polythene sleeve moist chamber 2.5 in. diameter and 0.0015 in. thick (Stanton, 1951). Areas of unrolled paper cylinders, bearing sporulating mycelium, were placed on glass slides and used as sources of inoculum, to avoid preparation of spore suspensions and possible damage to the conidia. Inoculation was achieved by spraying distilled water from a glass atomizer across the surface of the culture into the moist chamber, which was subsequently closed for 24 hr. The sleeve was then opened for 2 hr. to allow a gradual adjustment to less humid conditions and finally removed altogether. The degree of infection resulting from each combination of fungal clone and scion variety was recorded 30 and 50 days after inoculation. Symptoms of infection were divided into two main types:

(a) Fleck reaction, which was defined as a chlorotic spot with or without a necrotic centre and bearing no conidia.

(b) Lesion reaction, in which few or many conidia were produced.

During the summer months, adequate shading of the greenhouse was necessary to avoid sun scald, Cox's Orange Pippin being particularly susceptible. The trees were sprayed with water two or three times daily, depending on weather conditions, to control mildew (*Podosphaera leucotricha*). Infected leaves were removed when possible or the lesions were covered with traces of vaseline.

RESULTS

Sporulation and polymorphism in artificial culture

Fifty-one isolates of *Venturia inaequalis* were obtained from a wide range of host varieties in order to make a collection which, though small, would be as heterogeneous as possible and provide useful material for comparative studies of cultural characters and pathogenicity. The isolates could not be regarded as physiologically specialized strains and are therefore described as culturally distinct clones. As was expected from the strongly polymorphic nature of the fungus (Palmiter, 1934; Rudloff & Schmidt, 1934; Schmidt, 1935, 1936*a, b*) no two primary lesions yielded the same colony form, but replicate isolations from the same lesion produced colonies identical in appearance with only one exception.

Table 1. *Classification of colony characters of Venturia inaequalis and V. pirina*

Colony characters	Group		
	1	2	3
Size (diameter)	Small (< 12 mm.)	Intermediate or large	Large (> 20 mm.)
Macroscopic appearance of aerial mycelium	Rough and vertical	Intermediate	Smooth and horizontal
Predominance of subaerial or aerial mycelium	Subaerial (S) or aerial (A)	Subaerial or aerial	Aerial
Edge	Poorly defined	Intermediate	Well defined
Mycelium at edge	Hyphae undulating and profusely branched	Intermediate	Hyphae straight and unbranched
Sporulation at edge	High	Variable	Low
Colony type	1S or 1A	2S or 2A	3A

An attempt was made to simplify the description of, and to classify, the many morphological differences between the clones. Colonies were initially divided into three groups depending on the characters listed in Table 1. Predominance of subaerial or aerial mycelium on a standard agar depth was found to be variable within groups 1 and 2, so a further separation was made within each of these two groups. Thus five colony types were distinguished (1S, 1A, 2S, 2A and 3A) as shown in Table 1. The few mutant forms which appeared during the course of the cultural studies were, with one exception, invariably of the 3A type and usually sterile. The sporulation of each isolate on 10% (w/v) malt-extract solution was graded according to the magnitude of the turbidity estimate: grade 1 = > 0.2, grade 2 = 0.2-0.05 and grade 3 = < 0.05. The results suggest that there is a relationship between colony type and sporulation grade as summarized in Table 2.

Groups 1 and 3 seem to be composed of extreme types: thirty-four of the fifty-one clones belonged to group 2. No group 1 clones sporulated at a

Table 2. *The relationship between sporulation grade and colony type in Venturia inaequalis*

Colony type	No. of clones	Sporulation grade		
		1	2	3
		No. of clones		
1S	2	2	0	0
1A	6	6	0	0
2S	21	15	6	0
2A	13	2	7	4
3A	9	0	4	5

degree below grade 1 and no group 3 clones above grade 2. Group 2 was fairly easily divisible into 2S and 2A types, high sporulation often being associated with a predominance of subaerial mycelium. Growth on the paper cylinders was similar to that on agar; group 1 clones produced a thin mycelial cover and very many conidia, and group 3 clones a very thick mycelial cover and few conidia. Conidia appeared to be evenly distributed through each culture, in contrast to *Venturia pirina* (see below). The relationship between profuse vegetative growth and low sporulation seems to be due, at least partly, to rapid detachment and germination of the conidia, resulting in a thickening of mycelial cover. Nevertheless, large clusters of conidia were never seen in paper cylinder cultures of group 3 clones.

A similar relationship between sporulation grade and colony type was observed during an examination of thirty-seven clones of *Venturia pirina* which were isolated from various varieties. The colonies were classified into the five types described under *V. inaequalis*. As noted by Herbst (1936) relatively few conidia were produced in culture and the sporulation grades were therefore adjusted as follows: grade 1 = >0.02 , grade 2 = $0.02-0.005$ and grade 3 = <0.005 .

Maximal sporulation was again associated with a predominance of subaerial mycelium. The majority of clones belonged to group 2, but unlike *Venturia inaequalis*, a large number of 2S types sporulated at the grade 2 level. High sporulation in the 2S type was associated with minimal thickness of aerial mycelium and shades of dark brown or black in colony colour.

Table 3. *The relationship between sporulation grade and colony type in Venturia pirina*

Colony type	No. of clones	Sporulation grade		
		1	2	3
		No. of clones		
1S	2	2	0	0
1A	3	0	2	1
2S	17	6	11	0
2A	10	0	4	6
3A	5	0	1	4

Using malt-extract various attempts were made to increase the sporulation of a grade 1 clone of *Venturia pirina* on paper cylinders. The volume of medium was varied between 1 and 4 ml. and the extract was used at 2, 5 and 10 % (w/v). Some of the paper cylinders were rotated at intervals during incubation to expose the complete surfaces for development of aerial mycelium. Significant increases in sporulation were not observed. In contrast to *V. inaequalis*, conidia are formed only on the aerial mycelium and thus the amount of mycelium available for spore production in any given culture is far less in *V. pirina*. Growth on the paper cylinders was again similar to that on agar, thin dark layers of aerial mycelium being associated with maximal sporulation and vice versa. Gentle shaking with water was efficient for removal of conidia only from the cultures; more vigorous treatment resulted in a mixture of conidia and fragments of the strongly segmented subaerial mycelium.

Measurement of sporulation as a main cultural response is obviously less satisfactory for *Venturia pirina*. However, in order to obtain results comparable with those from studies of *V. inaequalis* and to investigate the possibility of stimulation of sporulation, this method was adopted. Apart from categorized growth estimates, no attempt was made to explore other methods of measuring reactions to *in vitro* conditions.

Nutritional requirements in artificial culture

Three culture media (Table 4) were used as standards and compared with modifications which are specified below. All media except malt-extract were adjusted to pH 5.6 by the use of dip-type electrodes, and therefore contained traces of potassium chloride. Small amounts of malt-extract were added with

Table 4. *Composition of the standard media used in studies of the nutritional requirements of Venturia inaequalis and V. pirina*

Constituent	Media		
	Dox	NC	10 % malt-extract
Glucose (g.)	1.5	1.5	—
NaNO ₃ (g.)	0.255	—	—
Casein digest (g.)	—	0.35	—
Thiamine (μg.)	—	30	—
MgSO ₄ .7H ₂ O (g.)	0.05	0.05	—
KH ₂ PO ₄ (g.)	0.15	0.15	—
Malt-extract (g.)	—	—	10.0
Trace element solution (ml.)	—	100	—
Distilled water (ml.)	100	—	100

the unwashed inoculum. All the constituents were included at the concentrations given in Table 4 unless otherwise stated (nitrogen = 420 mg./l.). 'Dox' is the modification of Dox's Solution used by Leben & Keitt (1948), but it was found desirable to increase the glucose concentration to 1.5 % (w/v). 'NC' refers to a partially defined medium containing all the known growth requirements of *Venturia* species. Sodium nitrate was replaced by an enzymic digest

of casein on a total nitrogen basis. Thiamine was included at the maximum concentration used by Leben & Keitt, and their trace element solution was also used (Zn, 90; Cu, 20; Mn, 10; B, 5; Mo, 10; and Fe, 100 $\mu\text{g./l.}$). The malt-extract was a crystalline preparation and as the pH value of the solution was 5.6 no adjustment was necessary.

The paper cylinder cultures were incubated until they were judged to be mature, i.e. to the stage just before the beginning of spore germination which resulted in thickening of mycelial cover and the adoption of a dry and woolly appearance. As a result of rapid growth of aerial mycelium on 10% malt-extract, the end-points of cultures on this medium were sometimes misjudged in the early tests (1-5) and so artificially low sporulation figures were recorded, the cultures being either too young or past the mature stage. The accompanying graphs (Figs. 1, 2) illustrate sporulation of *Venturia inaequalis*, clone E1,

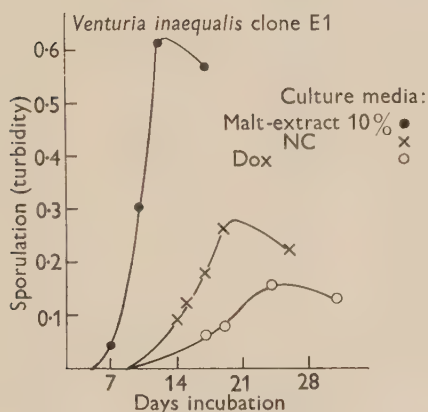


Fig. 1. Sporulation of *Venturia inaequalis* on three media at 18°.

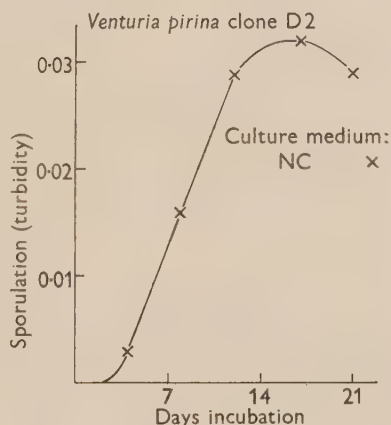


Fig. 2. Sporulation of *Venturia pirina* at 18°.

on the three standard media and of *V. pirina*, clone D2, on medium NC, and demonstrate the importance of taking times of maximum sporulation as cultural end-points.

Table 5 shows results obtained with *Venturia inaequalis*, clones E1 and C1 isolated from the varieties Edward VII and Cox's Orange Pippin respectively, and *V. pirina* clones D2 and Cf5 isolated from Durondeau and Conference respectively. Apart from the responses to urea as a nitrogen source, marked similarities were observed between the two species, and between clones of the same species which differed in other characteristics (see below). A very wide tolerance of nutritional conditions was suggested. Glucose with inorganic salts and a trace of malt-extract supported growth and sporulation, and thiamine, trace elements and casein digest appeared to be limiting factors in nutrition as found by Leben & Keitt (1948) in their studies of *V. inaequalis*. Maximum sporulation of *V. inaequalis* occurred on 10% malt-extract, and the sporulation of this species on the defined media was best in the presence of an organic nitrogen source. These results confirm those of Rudloff (1934a)

and Miller (1949). *V. pirina* differed from *V. inaequalis* in that maximum sporulation was not always associated with optimum growth. Rudloff (1934*a*) found that growth and sporulation of *V. inaequalis* varied independently, but the present observation that cultures of this species showing optimum growth yielded most conidia, does not imply that the number of conidia per unit volume of mycelium was the same in all cultures.

Table 5. *Growth and sporulation on various culture media**Venturia inaequalis*, clones E1 and C1; *V. pirina*, clones D2 and Cf5

Test no.	Culture medium	Sporulation			Days incubation			Growth category	
		E1	C1	D2	E1	C1, D2	Cf5	E1, C1	D2
5	10 % malt-extract	0.229	0.269	0.025	—	19	—	3	3
	Dox	0.056	0.068	0.016	—	19	—	1	1
	Dox + trace elements	0.060	0.085	0.018	—	19	—	1	1
	No. 3 + thiamine	0.138	0.072	0.011	—	19	—	1	1
	No. 4 with 3x NaNO ₃	0.115	0.060	0.004	—	19	—	1	1
	No. 4 with NaNO ₃ replaced by urea	<0.001	<0.001	0.034	—	19	—	0	2
	NC	0.389	0.275	0.025	—	19	—	2	2
	NC with sucrose, glucose and fructose each at 0.5 % (w/v)	0.389	0.170	0.019	—	19	—	2	2
		E1	C1	Cf5	E1	C1	Cf5	E1	Cf5
3	10 % malt-extract	0.309	—	0.007	12	—	18	3	3
	Dox	0.186	—	0.015	18	—	18	1	1
	Dox with sucrose, glucose and fructose each at 0.5 %	0.110	—	0.012	18	—	18	1	1
	No. 3 + thiamine and trace elements	0.269	—	0.027	18	—	18	1	1
	No. 4 with NaNO ₃ replaced by amino acids*	0.200	—	0.002	12	—	18	2	1
	No. 4 with NaNO ₃ replaced by urea	0.062	—	0.032	18	—	18	1	2
	NC with sucrose, glucose and fructose each at 0.5 %	0.427	—	0.011	12	—	18	2	2
		E1	C1	D2	E1, D2	C1	Cf5	E1, D2	D2
21	Dox	0.010	—	0.021	26	—	—	1	—
	Dox with NaNO ₃ replaced by casein digest	0.135	—	0.014	26	—	—	2	—

* L-asparagine, L-glutamic acid and L-valine each at 140 mg. N/l.

The effects of variation of the C:N ratio and of the concentrations of the carbon and nitrogen sources in medium NC, on the growth and sporulation of *Venturia inaequalis*, clones E1 and A7 (isolated from Worcester Pearmain) and *V. pirina*, clone D2, are shown in Table 6. Decrease of the casein digest concentration to one-third of the normal level is indicated as ' $\frac{1}{3}$ NC' and increase in glucose concentration as 'N 3C', etc.

As expected a high degree of tolerance to variation in concentrations was observed. Decrease of sugar concentration decreased the growth and sporulation of all three clones, and decrease of nitrogen decreased sporulation. Increase of carbon and nitrogen above the normal values had little effect on E1

and decreased sporulation of A7, while clone D2 showed a distinct preference for the C:N ratio of medium NC. Similarly, tolerance of trace elements up to twenty times the normal concentration was recorded, though this resulted in some diminution in sporulation of clones E1, A7 and *V. pirina*, clone P17 isolated from Williams pear.

Table 6. *Effects of variation in glucose and casein digest concentrations, in medium NC, on growth and sporulation*

Venturia inaequalis, clone E1, incubated 19 days; *V. inaequalis*, clone A7, incubated 24 days; *V. pirina*, clone D2 incubated 18 days.

Culture medium	Sporulation			Growth category	
	E1	A7	D2	E1, A7	D2
$\frac{1}{3}$ N $\frac{1}{3}$ C	0.105	0.041	0.028	1	1
$\frac{1}{3}$ NC	0.170	0.046	0.012	2	2
$\frac{1}{3}$ N 3C	0.123	0.021	0.007	2	2
N $\frac{1}{3}$ C	0.155	0.068	0.020	1	1
NC	0.263	0.085	0.044	2	2
N 3C	0.251	0.069	0.009	2	2
3N $\frac{1}{3}$ C	0.107	0.043	0.017	1	1
3NC	0.339	0.060	0.026	2	2
3N 3C	0.204	0.018	0.042	2	3

Pathogenicity and host range

The host range of a clone is defined as the degrees of pathogenicity observed on a series of host varieties graded according to disease resistance under field conditions. Miller's Seedling and Conference were included as resistant apple and pear varieties respectively, and Cox and Williams as less resistant, other varieties being initially classed as intermediates. Four replicate trials were planned, using six clones each of *Venturia inaequalis* and *V. pirina*, and were carried out as far as limitations of space and material permitted. In addition to the clones mentioned above, *V. inaequalis* clone A20, isolated from Laxton's Superb, A6 from Worcester Pearmain and A26 from Cox's Orange Pippin were tested, and also *V. pirina*, P6 from Laxton's Superb, P16 from Williams' Bon Chrétien and Cf3 and 81' from Conference. Owing to the use of surplus nursery material it was not possible to obtain all the apple varieties on Malling II. Some varieties were included on more than one rootstock to demonstrate any possible effects on resistance. In each experiment symptoms of infection were recorded 30 and 50 days after inoculation, using the following disease categories: 0=no reaction; 1=fleck; 2=lesion; 3=high incidence of disease with defoliation.

The result of the four trials in terms of the maximum category number observed in each combination of clone and variety are summarized in Table 7. Clone E1, originally isolated from Edward VII which is a comparatively resistant variety, had the widest host range on the graded series of apple varieties, while clones C1 and A26 produced lesions only on Cox and Newton. No symptoms of infection were observed on the highly resistant variety

Miller's Seedling and no distinct rootstock effects were recorded. In contrast to the reactions of clone E1 on the apple varieties, clones Cf3 and 81' from Conference pear, which was classified as a resistant variety in 1951, were least pathogenic on the pear varieties. All the clones of *Venturia pirina* were pathogenic on Conference, though disease incidence was generally light and symptoms were not visible by the thirty-day record. There was some indication that Williams pear was more susceptible on Quince A than on Quince C.

Table 7. *Host ranges of six clones each of Venturia inaequalis and V. pirina. Maximum disease category number observed in each combination of clone and variety*

Varieties arranged in increasing order of resistance.									
Clone of pathogen and variety of origin									
Apple variety	Rootstock	E1	A 20	A7	A6	A26	C1		
		Edward	Laxton's Superb	Worcester	Worcester	Cox	Cox		
		Cox's Orange Pippin	Malling II	3	3	3	2	3	2
		Cox's Orange Pippin	Malling IV	3	3	3	2	3	3
		Newton Wonder	Malling XVI	2	2	3	2	2	3
		Worcester Pearmain	Malling II	2	2	2	2	0	1
		Worcester Pearmain	Malling IV	3	2	2	2	1	0
		James Grieve	Malling VII	2	2	2	1	1	1
		Laxton's Superb	Malling II	2	2	1	1	1	1
		Edward VII	Malling VII	2	0	0	0	0	0
		Miller's Seedling	Malling IV	0	0	0	0	0	0
Pear variety	Rootstock	P 6	D 2	P 16	P 17	Cf3	81'		
		Laxton's Superb	Duron- deau	Williams	Williams	Con- ference	Con- ference		
		Williams' Bon Chrétien	Quince A	2	2	3	2	2	2
		Williams' Bon Chrétien	Quince C	1	2	2	2	1	1
		Doyenné du Comice	Quince A	2	2	2	2	2	2
		Laxton's Superb	Quince A	2	1	2	0	1	1
		Beurré d'Amanlis	Quince A	2	0	2	2	0	0
		Beurré Hardy	Quince A	2	2	1	1	1	1
		Beurré Hardy	Quince C	2	2	1	1	1	1
		Conference	Quince A	2	2	2	2	2	2

Stability in culture and the effects of re-isolation from artificial infection

Stability in culture is defined as the maintenance of unchanged sporulation grade, colony type and pathogenicity during storage at 5° on 2% malt-extract agar under paraffin oil. Six clones each of *Venturia inaequalis* and *V. pirina* were periodically re-isolated from inoculated shoots of Cox's Orange Pippin and Williams' Bon Chrétien respectively. In no case was any clone except the one used as inoculum recovered from a given tree, which showed that no spread of infection was occurring under the greenhouse conditions. The behaviour of the clones during storage periods of five years (E1, C1, D2 and Cf3) and four years (A6, A7, A20, A26, P6, P16, P17 and 81') is shown in Table 8. At the time of the first re-isolation, clones E1, C1, D2 and Cf3 had been stored for two years while the remainder had been isolated only one year previously. The time intervals between the four re-isolations were 1-2, eleven

Table 8. *Effects of periodic re-isolation from artificial infection on stability of Venturia inaequalis and V. pirina in artificial culture*

Clone of pathogen <i>V. inaequalis</i>	After re-isolation no.															
	1 (from host)				2 (from stock cultures)†				3 (from host)				4 (from host)			
	Colony type	Sporulation grade	Colony type	Sporulation grade	Pathogenic category	Colony type	Sporulation grade	Pathogenic category	Colony type	Sporulation grade	Pathogenic category	Colony type	Sporulation grade	Pathogenic category	Colony type	Sporulation grade
<i>V. inaequalis</i>	Apple varietal source															
	E 1	2S	1	1	3	2S	1	—	2S	1	2	2S	1	3	—	—
	A 20	2S	1	1	3	2S	2	—	2S	1	2	2S	1	3	—	—
	A 6	1A	1	1	3	1A	2	—	1A	1	2	1A	1	3	—	—
	A 7	2S	1	1	3	2A	2	—	2S	1	2	2S	1	3	—	—
	A 26	2S	1	1	3	2A	2	—	2A	3	2	—	—	—	—	—
<i>V. pirina</i>	C 1	2A	1	2	3	3A	3	—	—	—	—	—	—	—	—	—
	Pear															
	D 2	2S	1	1	2	2S	1	—	2S	1	2	—	—	1†	—	—
	P 6	2S	1	1	2	2S	1	—	2S	1	2	—	—	1	—	—
	P 16	2A	2	2	3	2A	2	—	2A	2	2	—	—	1	—	—
	P 17	1A	2	2	3	1A	2	—	1A	2	2	—	—	1	—	—
	81	2S	1	1	2	2S	1	—	—	—	—	—	—	—	—	—
	Cf 3	2S	2	2	2	2S	2	—	—	—	—	—	—	—	—	—

* At time of re-isolation.

† Owing to failure of trees.

† No re-isolation possible.

months; 2-3, seven months; 3-4, fourteen months. All the clones were isolated as single conidia from the stock cultures at the second re-isolation, owing to a failure of trees in the greenhouse. Consequently eighteen months elapsed between re-isolations 1 and 3 from artificial infection.

Venturia inaequalis showed a tendency to increased development of aerial mycelium associated with reduced sporulation and loss of pathogenicity on Cox. Clones E1 and A20, which had the widest host ranges, were the most stable, and C1 and A26 the least stable. All except the least stable clones regained their wild-type characteristics after re-isolation from Cox, and it is thought that the unstable types, C1 and A26, might have been retained in culture with frequent re-isolation. In contrast, no evidence was obtained of changes in cultural characters of *V. pirina*, but pathogenicity declined rapidly under the storage conditions. At the third re-isolation only a few scattered lesions were observed, and fourteen months later no sporulating lesions were produced and recovery of the clones was not possible. At the time of the host range trials (Table 7) the clones of *V. pirina* had been stored in culture for either one (P6, P16, P17 and 81') or two years (D2 and Cf3) and the results of these would therefore seem to indicate rather the maintenance of pathogenicity during the storage period than the actual host ranges of the wild-types.

DISCUSSION

The clones of *Venturia inaequalis* showed relationships between degree of resistance of host source, host range, and stability in culture. There was some indication that deviation from the wild type, observed as change in colony type or sporulation grade, indicated decreased pathogenicity on Cox, and the most stable clones had the widest host ranges on the varieties tested. No such relationships were observed in the study of *V. pirina*; in all cases cultural characters remained unchanged during prolonged storage, but pathogenicity declined. Nevertheless, variations in the maintenance of pathogenic capabilities on Williams were observed; thus *V. pirina* clone D2 was mildly pathogenic eighteen months after re-isolation, while another clone (not referred to in the text), isolated from Williams was non-pathogenic eleven months after isolation. There was also some indication of specialization of the Conference clones on Conference, as found by Stanton (1953*b*). Thus, in this instance, clones isolated from a variety which was regarded as resistant in 1951, had the narrowest host range.

Previous workers have not found these differences between *Venturia inaequalis* and *V. pirina*, though few have carried out parallel experiments on the two species. Herbst (1936), Langford & Keitt (1942) and Stanton (1953*a, b*) found their isolates of *V. pirina* to be culturally stable, apart from the occasional production of sectors. The English and American workers also found no relationship between host source and host range in *V. pirina*. Similarly, K  the (1935), Rudloff (1934*a*), and Keitt & Langford (1941) stated that their isolates of *V. inaequalis* showed unchanged colony characters and pathogenic capabilities after prolonged storage on natural media, though they did not use the

paraffin oil method. Palmiter (1934) and Schmidt (1936*b*) also found no relationship between host source and host range in this species.

A larger number of clones would have to be examined before the relationships outlined above could be definitely established, and before differences between the species, in respect of these relationships, could be fully evaluated. There appear to be several factors of importance in this apparent discrepancy with the results of previous workers. The six clones of each species, which were selected for detailed study, had been collected from series of commercially important varieties varying as widely as possible in their degree of resistance to scab. This variation occurs to a considerably greater extent among apple varieties than among pear varieties commonly grown in England. Therefore, the wider divergence in the clonal characteristics of *Venturia inaequalis* is, perhaps, not unexpected. Clone E1, from the resistant variety Edward VII, was obtained only after prolonged search, while isolates were easily obtained from Conference, which was classified as a resistant variety when the isolations were made. It must however, be remembered that clone E1 has a wide host range and therefore might have been isolated from Cox, in which case the relationships described above would not have been apparent. Furthermore, as varietal resistance varies with locality, it could not be stated that, for example, isolates from Edward VII would invariably show characteristics similar to clone E1. It is suggested that the percentage of E1-type isolates obtained from an apple variety which is highly resistant in a given locality, may be greater than the percentage obtained from less resistant varieties in the same locality.

In both species of *Venturia* a relationship was established between colony type, which is genetically determined in the ascus, and sporulation on an optimum medium. The results confirm those of Rudloff (1934*b*) and Herbst (1936) and also show that maximum sporulation appears to be associated with minimal development of aerial mycelium. There were no relationships within the species either between cultural characters and stability in culture or, in accordance with the results of the German and American workers, between cultural characters and host range or host variety. Thus, the choice of grade 1 clones, which are best suited to studies of sporulation, has not prejudiced the inclusion of widely differing types among those selected for detailed study. It is suggested that, in the choice of clones of *V. inaequalis* as routine sources for large numbers of conidia over long periods, due account should be taken of the characters and relationships outlined above. Yearly re-isolation from artificial infection may be necessary to maintain the wild clonal type, as monoconidial transfer from the stock cultures in no case induced a return to normal. Clone E1 of *V. inaequalis* has been successfully used in these laboratories for spore germination tests of protective fungicides during the period 1949–56 and is unchanged in all observed characteristics. The use of *V. pirina* as a routine source of conidia presents a more difficult problem because of the low degrees of sporulation observed in culture. Choice of clone must be based on sporulation grade and frequent re-isolation seems to be necessary for the maintenance of the wild pathogenic type.

Apart from response to urea, no major differences in nutritional requirement were observed either between the species or between clones that differed widely in other characteristics. Even though differences have been found by Rudloff (1934*a*), Leben & Keitt (1948), Pelletier & Keitt (1954) and Fothergill & Ashcroft (1955) no attempt has been made to assess the importance of variation in the metabolism of varieties, characterized by different degrees of resistance, in the host relations of the pathogens.

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The Infection of Clover Root Hairs by Nodule Bacteria Studied by a Simple Glass Slide Technique

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SUMMARY: A simple glass slide technique has been devised for the continuous microscopical observation of growth and infection of root hairs of clover seedlings. The method involves an aseptic cultivation of seedlings on microscope slides which are partly immersed in a mineral salts medium. The roots are protected by a cover-slip.

By this procedure, the root hairs of white clover inoculated with nodule bacteria were studied. The earliest infection was observed to take place within 48 hr. of inoculation, on 4-day-old seedlings. In branched hairs the growth of the thread from a lateral branch towards the hair tip is tentatively explained as an effect of the position of the hair nucleus relative to the site of infection.

The infection of leguminous plants by nodule bacteria has, as a rule, been studied with fixed and sectioned material. The first stages of this process may also be studied *in vivo*, but no suitable technique has yet been described for a continuous microscopical examination of these stages.

A method for the study of growing root hairs (of wheat) was devised by Lundegårdh (1946) and modified by Ekdahl (1953). In their work, a chamber containing flowing salt solution and resting on a microscope stage was employed. However, difficulties arise with this method if aseptic conditions are also necessary. The present author has therefore used a technique for the aseptic cultivation of young seedlings on microscope slides in a manner which permits periodic observations under the microscope of the growth and infection of individual root hairs. The arrangement also offers good conditions for photomicrography.

The details of the method are as follows.

METHOD

A nitrogen-free mineral solution of the following composition was prepared: CaCl_2 , 0.1 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12 g.; KH_2PO_4 , 0.1 g.; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.15 g.; Fe citrate, 0.005 g.; Mn, Cu, Zn, B, Mo traces; dist. water, 1000 ml.; pH 6.5 (after autoclaving).

Portions (25 ml.) of this solution were distributed into large glass tubes (39×125 mm.). The tubes were plugged with cotton wool or covered with closely fitting glass caps (45×60 mm.) and were sterilized in the autoclave for 20 min. at 120° (15 lb./sq.in.). Microscope slides (26×75 mm.) and cover-slips (24×40 mm.) were sterilized by heating dry in Petri dishes, preferably only one slide and one cover-slip in each dish.

Seeds of white clover (*Trifolium repens*, variety Morsö, from Svalöf, Sweden) were disinfected by successive treatment with 95 % ethanol and equal parts of 0.2 % formaldehyde and 0.2 % HgCl_2 and were then washed in several changes of sterile water. They were allowed to germinate at room temperature in a shallow layer of water.

After 2 days, when the seedlings were 10–15 mm. in length, they were transferred to the glass slides in the following manner: 6–7 drops (c. 0.2 ml.) of 0.3–0.4 % Bacto agar in the above-mentioned mineral solution, previously melted and cooled to c. 30°, were pipetted over one half of each slide, and immediately thereafter a seedling was taken with a platinum wire and placed on the slide with the root tip in the agar. The cover-slip was then laid carefully over the agar and the root by means of sterile forceps. The seed coat was removed, if it still adhered to the cotyledons. When all slides were prepared, they were transferred from the dishes into the tubes containing mineral solution.

As a rule, inoculation was made with a few drops of a thick suspension of young *Rhizobium trifolii* (local strain No. 220) added to the solution in the tubes. When rapid infection was desired, the agar was inoculated before making up the slides. All manipulations were made aseptically with sterile instruments in a sterile room.

The tubes were placed in a constant temperature room at 25° under a fluorescent lamp. For convenient handling the tubes were kept in a metal rack (in which they were also sterilized).

For the microscopic examination, the slides were taken from the tubes with forceps and excess solution drained off carefully with filter-paper. As an immersion liquid for the high-power objective glycerol instead of ordinary immersion oil was used, since it is easier to rinse off in water. After the examination, the slides were put back into their tubes.

For the observation of living plants and bacteria, the phase contrast equipment was employed. This facilitated the study of bacterial motion and protoplasmic streaming and enabled the nuclei and other structures of the root hairs to be readily observed, particularly in the longer hairs, at some distance from the main root.

Stained preparations were made by lifting off the cover-slip and immersing the slide in the dye solution for a suitable period of time. After this the root was covered with some drops of nutrient solution and a new cover-slip applied. As a rule, the root hairs earlier studied *in vivo* were easily found on renewed examination.

In the microscopic work, a Zeiss Lumipan microscope with a graduated mechanical stage was used. Photomicrographs were taken with the aid of a Busch Akaflax camera provided with 9 × 12 cm. glass plates, using Kodak O 120 plates.

The technique outlined above is simple and requires no special equipment. Its chief advantage is the possibility of making continuous observations on the development of the root hairs and on the activity of the infecting bacteria without disturbing the root system.

Some definite limitations of the technique should be pointed out. First, the

method is only suitable for small seedlings. Further, although initially under aseptic conditions the slides are intermittently exposed to air-borne bacteria and moulds. Experience has shown, however, that contaminating micro-organisms do not appear in the microscopic fields until many days after the first examination. This is probably because the medium is unsuitable for the rapid growth of most contaminants.

A third objection which might be put forward is that the limited space between the slide and coverslip does not permit as luxuriant a growth of the plant as under ordinary culture conditions. The development of individual root hairs is naturally affected by their position relative to the glasses. Any hair reaching the glass will be more or less deformed, and it is important to distinguish this merely physical deformation from that caused by infection.

RESULTS

With the method just described, the growth of seedlings of white clover was examined. The main results may be summarized as follows.

Time of infection

In experiments with lucerne plants, Thornton (1929, 1954) found that the infection, as manifested by the appearance of the first nodule, coincided with the opening of the first true leaf. This also holds true for clover (Chen & Thornton, 1940), but not necessarily for other plants. For example, Bieberdorf (1938) found no such correlation in soybeans. In red clover, Nutman (1946, 1953, 1956) observed much genetically-determined variation in this respect. In early nodulating strains, he obtained nodulation when the seedlings were 10–15 days old. Clearly in these cases the actual infection must precede nodulation by one or more days; however, Chen & Thornton (1940) remark that 'Nodules... appeared on the same day in which infected root hairs were first observed'.

In the writer's experiments also, great variation with regard to time for infection was observed. This is probably sufficiently explained by the heterogeneity of the plant material. In many plants, an infection of the root hairs was observed on the second day following inoculation (4 days after germination of the seed), at which time only the cotyledons had developed, whereas in other plants no infection was observed until the tenth day.

On the other hand, nodulation was never observed until 7–8 days after inoculation, even if root hair infection had occurred as early as on the second day. This may mean that the penetration of the root tissue required more time in these experiments than in those reported by Chen & Thornton. In confirmation of earlier experience, it was found that only a small proportion of the root hairs (less than 2%) became visibly infected.

Association of the rhizobia with the root hairs

Before infection, some sort of attachment of the nodule bacteria to the root hair surface may be expected. However, there was never any conspicuous accumulation of bacteria on those root hairs which were visibly infected. The

invasion of nodule bacteria between the root hairs could easily be followed in the microscope. These showed vigorous motility, but very few became attached to the hairs. Bacterial masses like the one pictured by, for example, Viernann (1929) could only be seen in old stages.

Other workers have observed small clumps of nodule bacteria attached to the root hairs (Thornton, 1936, in lucerne), which were again larger than the colonies observed at the tip of infected clover root hairs in the present investigation. The latter were only seen at the tip of some straight infected hairs, where no curling prevented the examination, and consisted of a few minute rods. The exact size of these rods has not been determined, but certainly their thickness did not exceed 0.2μ . Careful examination failed to show any rods of ordinary size at the point of infection.

In summary, it seems that a very restricted number of minute bacteria attack the root hairs and become the infecting agents. The probable importance of such small rods in the infection process was clearly recognized in the early work of Beijerinck (1888) and has also been commented upon recently by Nutman (1956).

Mechanism of entry

Several workers have observed a 'brilliant spot' and/or a 'bladder-like swelling' at the site of infection (Ward, 1887; Prazmowski, 1890; Dawson, 1900; Viernann, 1929). A more or less pronounced bud-like outgrowth, sometimes highly refractile, has frequently been noted also in the present investigation (Pl. 1, fig. 2, 3).

As an interpretation of this structure, it might be suggested that the nodule bacteria cause a local weakening of the cell wall, whereupon the osmotic pressure of the cell sap forces the wall to expand. This requires some kind of enzymic action, and although the thorough investigation of McCoy (1932) failed to demonstrate the production of cell-wall dissolving enzymes by the bacteria, this question is not yet finally settled. The possibility that the root hairs, in response to some stimulation by the bacteria, locally secrete an enzyme facilitating the penetration might also be considered. This idea seems to deserve some attention, especially since Neely, Ball, Hamner & Sell (1950) and Bryan & Newcomb (1954) have shown that the pectin methylesterase activity of some plant tissues is increased by auxins, for instance indole acetic acid, which is believed to be produced by nodule bacteria (Thornton & Nicol, 1936; Thimann, 1936; Chen, 1938; Georgi & Beguin, 1939).

The infection thread

There is general agreement that the nodule bacteria pass down the root hairs within an infection thread. This structure has always aroused the interest of those studying the interaction between nodule bacteria and legumes. Although this thread was observed and depicted by early investigators such as Eriksson (1874), Ward (1887) and Prazmowski (1890) its real nature and functioning is still debated, which is evident from the discussion of this subject

in the recent survey of Nutman (1956). The appearance of the infection thread as seen with phase contrast is shown in Pl. 1, fig. 4-6 and Pl. 2, fig. 7, 8.

The site of infection is always characterized by an intense plasmatic activity, the cell protoplasm and the cell nucleus being concentrated to this region (Pl. 1, fig. 4 and Pl. 2, fig. 7). This activity may be observed before any infection thread is visible, and it seems probable that the infection has then taken place, although no rigid thread structure has been formed (Pl. 3, fig. 13). The host plant may react by forming a transverse cell wall, although this does not prevent the further growth of the infection thread (Pl. 1, fig. 4-6).

From Pl. 2, fig. 7, it is evident that the tip has a round shape, a fact which is very difficult to establish with bright field illumination owing to the thick layer of protoplasm surrounding the tip. This may explain why early investigators like Frank (1890) and Dawson (1900) believed the end to be 'open'.

In the root hairs examined there was rarely more than one infection thread in each hair. Two threads were seen occasionally similar to those pictured by Bieberdorf (1938) in soybeans, but multiple threads as described by McCoy (1929) in *Phaseolus* were never encountered.

The growth rate of the infection threads varied to some extent. In some plants it was determined at a rate of between 5 and 8 μ ./hr. With a root hair length of 100-200 μ ., this would mean that an infection thread reaches the base of the hair within one day or slightly more. This result seems to agree with earlier observations on various legumes. As mentioned above, nodule formation was always delayed for many days.

Frequently, the growth of the infection threads stopped at an early stage (Pl. 1, fig. 5, 6). The proportion of unsuccessful threads is difficult to establish but was seldom less than 10-20 %.

Early investigators reported the occurrence of numerous bacterial rods within the infection threads (Prazmowski, 1890; Dawson, 1900; later Bieberdorf, 1938), while some more recent work suggests the existence of a single line of bacteria only (McCoy, 1929; Schaede, 1940). These observations were made on fixed and stained material, because it is almost impossible in unstained preparations to distinguish the bacteria from particles in the protoplasm surrounding the thread. This applies equally to observation with phase contrast, although the bacteria outside the root hairs are easily observed (Pl. 2, fig. 8).

After staining with suitable dyes the infection threads appear granular (Pl. 2, fig. 9). It is, however, doubtful whether all the minute stained particles are bacteria. The investigations of McCoy (1932) and Schaede (1940, 1941) have given convincing evidence that the infection thread soon becomes enclosed within a sheath of cellulose which, at least at the time of formation, is covered by a layer of living protoplasm. In living preparations small protoplasmic granules may be seen moving along the thread surface, and, in the present writer's opinion, most of the minute particles in stained preparations are on the outside of the thread.

Infection of branched root hairs

It was frequently observed in the course of the present work that infection threads were initiated in a branch or a bulb emerging from the side of a root hair, sometimes rather close to the base of the hair. It has not been definitely established whether such hairs have been infected at an early stage and then a new tip has been formed or whether the branches are themselves formed on fully grown hairs.

The infection threads originating in a side branch normally grow into the main part of the hair and turn down into the hair base. In many instances, however, such threads grew in the opposite direction, towards the apex of the hair (Pl. 2, fig. 10–11 and Pl. 3, fig. 12, 14). Such threads often ceased their growth before reaching the hair apex (Pl. 2, fig. 10), but occasionally a thread would turn near the tip and then grow back through the entire hair and disappear into the root tissue. In such cases double threads could be seen in the apical part of the hair, one ascending and one descending (Pl. 2, fig. 11).

An influence from the cell nuclei on the infection threads penetrating the root cortex was supposed by Beijerinck (1888). This was confirmed later by some authors (Dangeard, 1926; Schaede, 1940), but has been questioned by others (Dawson, 1900).

Observations of infection threads in branched hairs strongly suggested that the growth of the thread towards the hair tip was due to directing influence of the host cell nucleus. Abnormal threads were nearly always associated with the occurrence of the nucleus in the apical part of the hair (Pl. 3, fig. 12–14) whereas normal basipetal growth was associated with the location of the nucleus in the basal part of the hair. The very few exceptions to this rule were no doubt due to movement of the nucleus subsequent to the completion of growth of the infection thread.

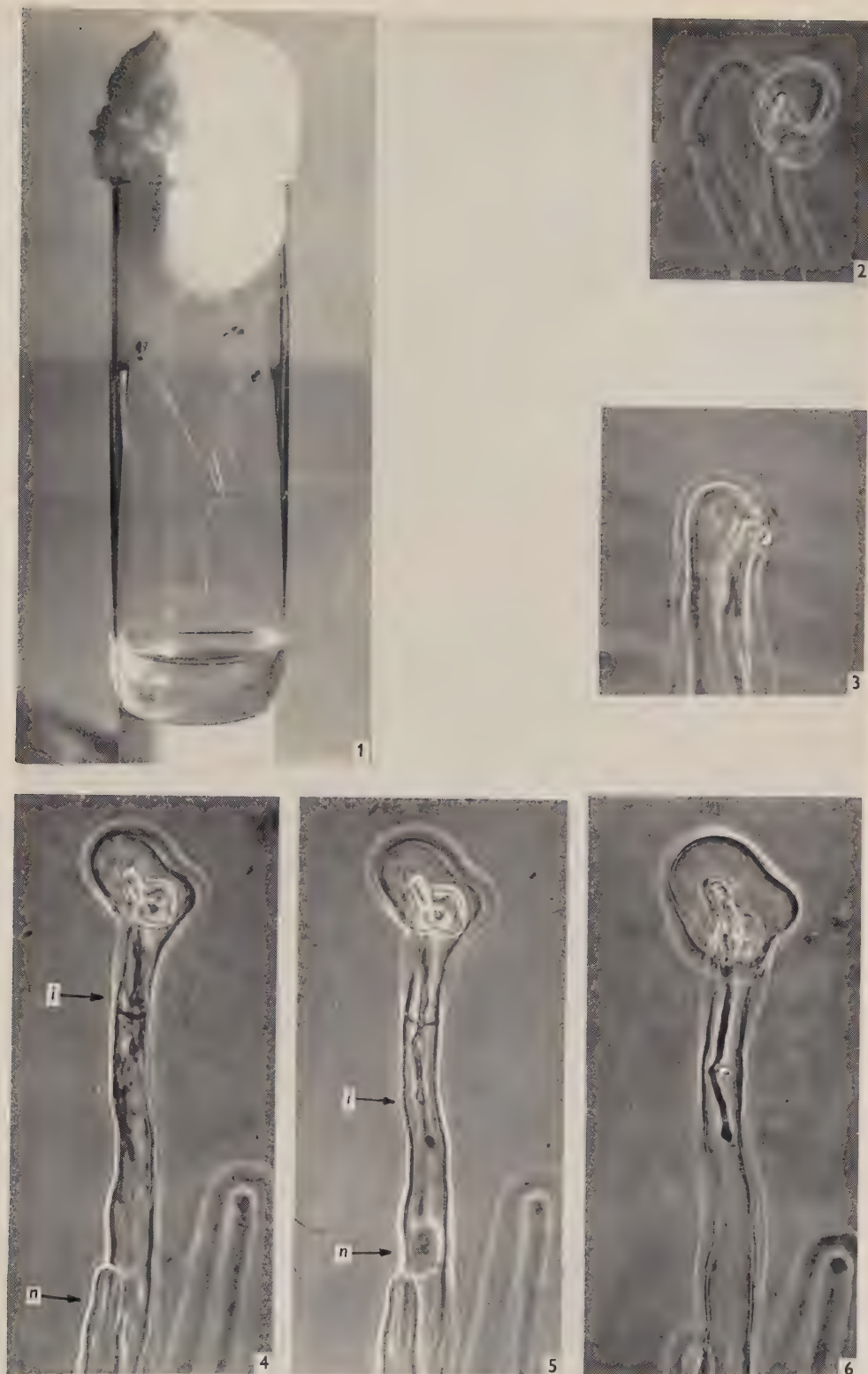
It appears that when growth in a certain direction is once initiated, the infection thread follows a straight course which is independent of slight fluctuations in the position of the nucleus. Some observations indicate that the nucleus may degenerate and assume a round shape as a result of the action of the infection thread.

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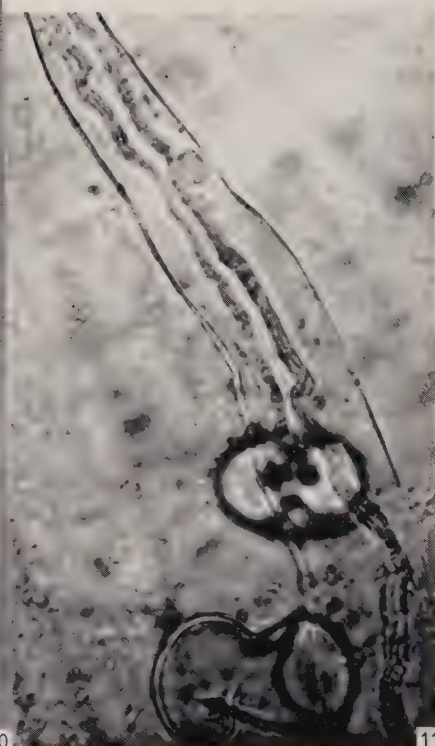
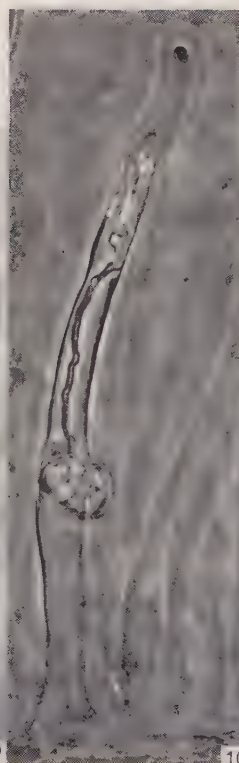
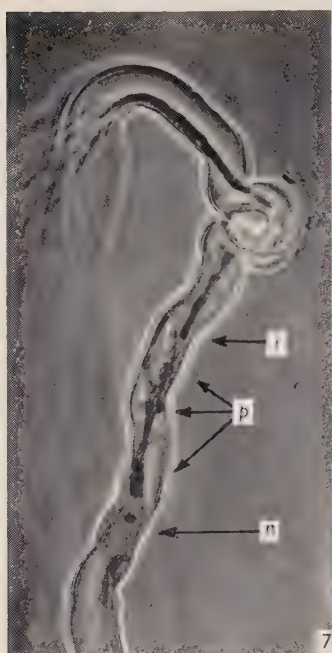
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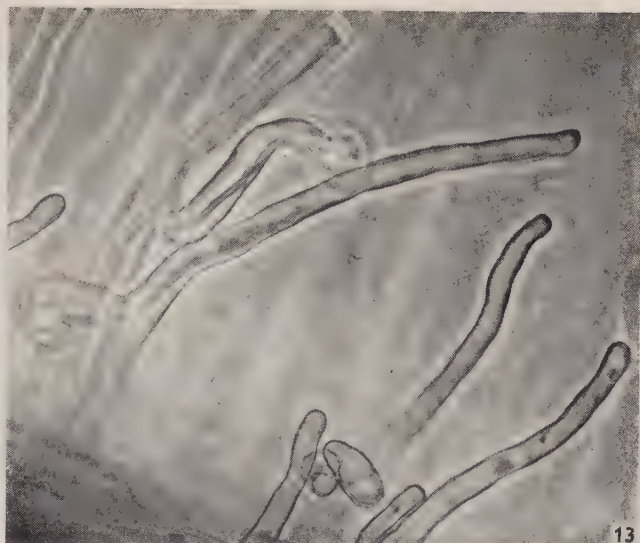
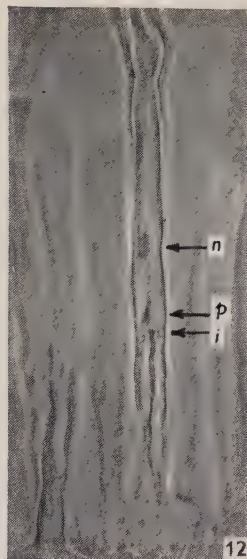
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G. FAHRAEUS--CLOVER ROOT INFECTION BY RHIZOBIA. PLATE 1

(Facing p. 380)





G. FÄHRÆUS—CLOVER ROOT INFECTION BY RHIZOBIA. PLATE 3

EXPLANATION OF PLATES

In all plates, *i*=tip of infection thread, *n*=nucleus, and *p*=protoplasm of root hair.

PLATE 1

- Fig. 1. Culture tube containing slide with a seedling a few weeks old. Nodules have appeared under the cover-slip. $\times 0.6$.
Fig. 2. Curled and infected hair with refractile emergence near the tip. Phase contrast, $\times 800$.
Fig. 3. Straight and infected hair with a similar structure. Phase contrast, $\times 900$.
Figs. 4-6. A single root hair photographed 4, 5, and 11 days after inoculation. Phase contrast, $\times c. 600$. The tip of the infection thread is almost concealed behind the streaming protoplasm in fig. 4 (4 days). Observations on the day before showed no infection thread, but intense plasmatic activity. No transverse wall had been formed and the nucleus was then located at *i* in Fig. 4. The wall has later degenerated (Fig. 6).

PLATE 2

- Fig. 7. Two hairs bent together, both infected. Photographed 48 hr. after inoculation. Phase contrast, $\times 630$.
Fig. 8. Infected root hair and bacterial rods outside the hair. Phase contrast, $\times 630$.
Fig. 9. Root hair with infection thread stained with methylene blue. Photographed in bright field, $\times 900$.
Fig. 10. Infection thread growing from side bulb towards the hair tip. Degenerated nucleus visible just above the entrance bulb. The thread has stopped growing and shows irregular swellings. Phase contrast, $\times 400$.
Fig. 11. Infection thread which has turned near the hair tip and grown back towards the base passing the entrance bulb. Stained with methylene blue, photographed in bright field, $\times 900$.

PLATE 3

- Fig. 12. Infection thread growing towards the hair nucleus. Phase contrast, $\times 280$.
Fig. 13. Two adjacent root hairs, one with a normal infection thread, the other probably infected and with actively motile protoplasm in the hair above the side bulb. The nucleus is situated in the protoplasmic strand. 4 days after inoculation. Phase contrast, $\times 400$.
Fig. 14. The same microscopic field, 19 hr. later. Three infection threads arising in hair branches, one growing normally into root and two growing towards hair tip. Both of the latter have stopped growing. Phase contrast, $\times 400$.

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The Distribution of Flagella in Dividing Bacteria

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SUMMARY: Two strains of bacteria which produced flagella when grown at 36° but not at 44° were examined; one was a strain of *Salmonella typhimurium* and the other of *Proteus vulgaris*. These organisms were grown on membranes for electron microscopy, being incubated at 36° so that the parent bacteria of each microcolony possessed a normal quota of flagella, and then transferred to 44° so that no more flagella were produced. In the microcolonies, after several divisions, it appeared that all these flagella were retained by the original parents, in accordance with the theory that bacteria of this type divide by budding from a growing-point at one pole.

The suggestion that the main growth of the cell envelopes in unicellular bacteria is from a growing-point at one pole was deduced from the behaviour of the flagella in dividing bacteria by Bisset (1951). It was found that in some cases one daughter had few flagella or none, whereas the other had a full complement; in other cases there appeared to be a progressive diminution in degree of development and age of the flagella from the point of division to the tip of one daughter, whereas once more the other daughter had all its flagella fully developed. At the putative growing-point the cell wall also appeared to be thinner and less well developed than over the remainder of the cell, and it could be shown to contain a relatively high concentration of basophilic material, presumably nucleoprotein.

Further evidence in support of this concept has been obtained from a variety of sources. Bergersen (1953) described the behaviour of similar growing points in *Escherichia coli* when their polarity of growth was so disturbed by cultivation in the presence of chloramphenicol that side-branches were produced instead of polar buds. Malek, Voskyova, Wolf & Fiala (1954), by means of cinematography, confirmed the concept that bacteria of this type grow from one pole. The corollary, that multicellular septate bacteria grow from the points of junction of cell wall and cross-wall, i.e. from the tips of each component cell (Bisset, 1953) was confirmed by Tomesik (1956) with his elegant micro-serological technique.

However, it was suggested by Stocker (1956) and by Quadling & Stocker (1956) that growth of the cell wall is diffuse and not localized at a growing point. These authors based their conclusions mainly upon the behaviour of a strain of *Salmonella typhimurium* which produced flagella at 37° but not at 44°. Cultures were grown for a period at 37° and then transferred to 44° so that no more flagella were produced, although growth and division continued. When examined by Leifson's flagellar stain, the appearance of these cultures suggested that the flagella had not been retained by a small number of bacilli, representing those which already possessed them at the end of their period of growth

at 37° in accordance with the growing point theory, but that they were distributed throughout the culture.

In an attempt to resolve the discrepancy between these two sets of observations, it was decided to attempt to repeat those of Quadling & Stocker upon *Salmonella typhimurium* by means of electron microscopy, which it was believed might provide a more reliable picture of the arrangement of the flagella than do the accretive staining methods. Similar observations were made upon a strain of *Proteus vulgaris* which behaved similarly in that it produced flagella when grown at 36° but not at 44°.

METHODS

The strain of *Salmonella typhimurium* used in his own experiments was kindly supplied by Dr B. A. D. Stocker (Lister Institute, London). It was originally intended that a series of electron micrographs should be made upon material prepared by Drs Quadling and Stocker exactly in the same manner as they had done previously, but these cultures unfortunately proved to be mucilaginous and generally unsuitable for electron microscopy. Accordingly, it was decided to grow the bacteria upon collodion membranes over meat infusion agar according to the method of Hillier, Knaysi & Baker (1948), and by inoculating with a very dilute suspension, to endeavour to obtain isolated microcolonies grown from one or a very small number of individuals in the inoculum. The inoculated membranes were incubated at 36° for periods of from 1 to 4 hr. and then transferred to 44° for further periods of one or more hours; they were then examined under the electron microscope.

These experiments were repeated with a freshly isolated strain of *Proteus vulgaris*, which was the most satisfactory of several strains of Gram-negative bacteria found to behave similarly with respect to the production of flagella at different temperatures. Other organisms proved liable to lose their flagella permanently, or their reactions were less-clear cut.

RESULTS

Whenever conditions were so arranged that discrete microcolonies were obtainable on the grids for examination, their appearance was entirely compatible with the growing-point theory of Bisset (1951). Only one, or a very small number of bacteria in each group appeared to possess flagella, and in these cases they had from six upwards; the majority had none at all. Thus it appeared as if during the initial period of incubation at 36° the flagella had developed normally, and that on transfer to 44° their development had ceased. The bacteria, however, had continued to divide at the higher temperature, and had grown from one pole, so that all the flagella remained with one daughter, the other being a naked bud.

These results are shown in Pl. 1, figs. 1-3, and explained in Figs. 1 and 2. Pl. 1, fig. 1, shows a portion of the circumference of a colony of *Salmonella typhimurium* grown from a fairly heavy inoculum which, after 3 hr. at 36° and then 1 hr. at 44°, produced some colonies and some areas of confluent growth,

it being assumed that the latter area developed from the confluence of several microcolonies. At the edge, where some flagella can be discerned, three bacilli only have flagella (arrows), the remainder have none. The unattached whips are presumably derived from the flagellate organisms of the inoculum. Pl. 1, figs. 2, 3 and 2*b*, 3*b* show microcolonies of *Proteus vulgaris*, grown from dilute inocula for 1 hr. at 36° and 2 hr. at 44°. One cell in each case bears a full complement of flagella, the rest have none, although some have the short filaments known as 'fimbrae'. In several hundred similar microcolonies, examined upon some twenty grids derived from a series of such experiments, exactly similar results were seen in every case. These observations are in accordance with the theory of bacterial growth from a growing point at one pole (Bisset, 1951). The reason for the discrepancy between these results and those of Quadling & Stocker (1956) remains unexplained, despite the valued co-operation of these authors in the present investigation.

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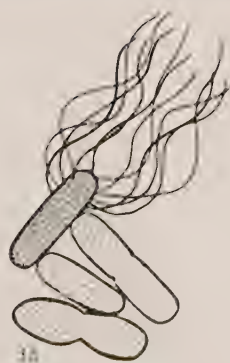
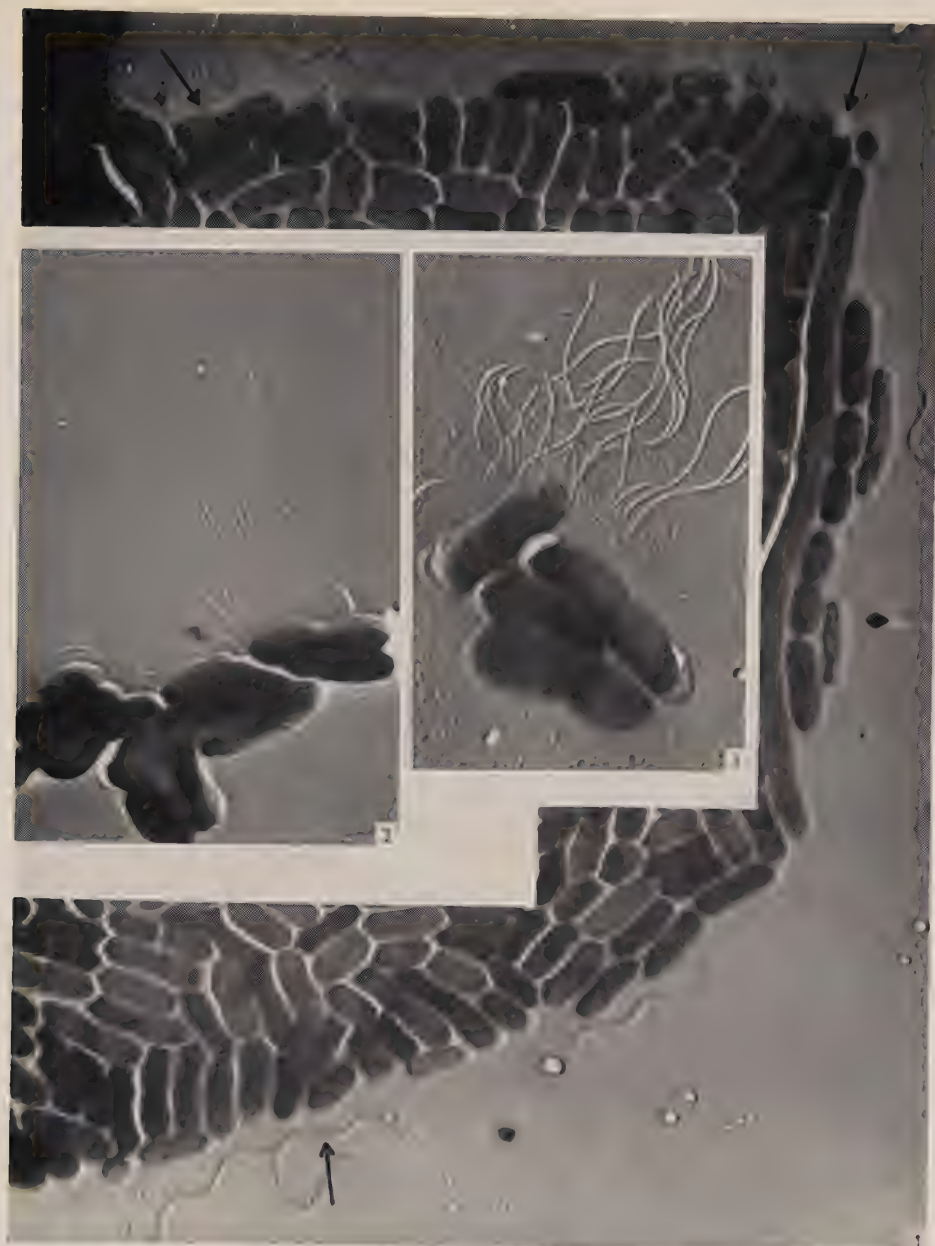
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EXPLANATION OF PLATE

PLATE 1

- Fig. 1. *Salmonella typhimurium*. Grown for 3 hr. at 36° then 1 hr. at 44°. Edge of colony; flagellate cells shown by arrows. Electron micrograph, metal shadowed; $\times 6000$.
- Figs. 2, 3. *Proteus vulgaris*. Grown for 1 hr. at 36° then 2 hr. at 44°. Microcolonies; one cell in each colony has numerous flagella; some of the remainder have 'fimbrae'. Electron micrograph, metal shadowed; $\times 8000$.
- Figs. 2*b* and 3*b* are drawn from Pl. 1, figs. 2 and 3 respectively. They show microcolonies after incubation for 1 hr. at 36°, to permit development of flagella in the germinating inoculum, followed by 2 hr. at 44°, when no further flagella should have developed. In each case, only one cell has flagella.

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K. A. BISSET AND P. PEASE—THE DISTRIBUTION OF FLAGELLA IN DIVIDING BACTERIA.
PLATE I

UDEN, N. VAN & L. DO CARMO SOUSA. (1957). *J. gen. Microbiol.* **16**, 385-395

Yeasts from the Bovine Caecum

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SUMMARY: From the caeca of 252 adult bovines 131 yeast strains were isolated, distributed as shown (in brackets) among the following species: *Saccharomyces cerevisiae* (12), *S. chevalieri* (3), *S. drosophilae* (3), *S. fragilis* (1), *Pichia bovis* (1), *P. membranaefaciens* (3), *P. fermentans* (3), *Cryptococcus diffluens* (1), *Torulopsis glabrata* (4), *Torulopsis* sp. (2), *Candida tropicalis* (45), *C. parapsilosis* (3), *C. krusei* (33), *C. macedoniensis* (1), *C. utilis* (1), *C. bovina* (1), *Trichosporon cutaneum* (5). *Candida albicans* was not isolated. The apparent absence of *C. albicans* from the bovine intestinal tract may explain the rareness of oral and intestinal moniliasis in bovines. Infection in certain forms of yeast mastitis (caused neither by *C. albicans* nor by *Cryptococcus neoformans*) may be derived from the intestinal tract.

Descriptions and Latin diagnoses of *Pichia bovis* n.sp. and *Candida bovina* n.sp. are given.

Knowledge of the intestinal yeast flora of man and animals has both ecological and medical importance. Some of the more important ecological aspects so far brought to light may be summarized as follows. Certain yeasts have their principal habitat in the human or animal digestive tract and have never, or only exceptionally, been found outside the human or animal body. Within this group several species can be isolated from many different animals (*Candida albicans*, *Torulopsis glabrata*, etc.), while others seem peculiar to a few host species (*Saccharomycopsis guttulata*, *Torulopsis pintolopesii*, etc.). A second group is constituted by yeasts which have their natural habitat on a wide variety of substrata including the human and animal digestive tract (*Candida tropicalis*, *C. krusei*, *Trichosporon cutaneum*, etc.). The third and by far the largest group contains yeast species which are not true inhabitants of the digestive tract, but which may remain viable for some time after entering the digestive tract with food or otherwise (*Saccharomyces cerevisiae* and many others).

From the medical and veterinary point of view knowledge of the intestinal yeast flora of man and animals is highly desirable, as it may elucidate the mechanisms of infection and re-infection in certain yeast diseases. It is now clear, for example, that moniliasis of man and certain birds is an endogenous mycosis, i.e. the causative yeasts (mostly *Candida albicans*, rarely *C. tropicalis* or others) live saprophytically in the digestive tract and may, under special conditions, invade various organs and cause disease.

In the present study an attempt has been made to determine the bovine intestinal yeast flora for a double purpose: (1) to contribute to the knowledge of the ecology of human and animal yeasts; (2) to obtain information on the possible relationship between the bovine intestinal yeast flora and bovine yeast diseases, particularly yeast mastitis.

METHODS

Two hundred and fifty-two adult bovines were sampled in the abattoir of Lisbon. Immediately after evisceration a small area of the surface of the caecum was cleaned with ethanol and incised with flamed scissors. Then a loopful of faecal material was inoculated in a tube containing broth of the following composition: glucose, 2%; yeast extract (Difco), 0.5%; peptone, 1%; distilled water. To limit bacterial growth 50 units penicillin/ml. and 100 units streptomycin/ml. had been added to the broth. To exclude all yeasts which do not thrive at body temperature and therefore cannot be considered intestinal inhabitants, the tubes were incubated at 37°. Within 3–5 days most tubes showed growth of filamentous fungi, mostly species of the genera *Aspergillus*, *Mucor*, *Rhizopus* and *Absidia*. As the conidia and sporangiospores of these fungi would make it very difficult to isolate any yeasts present in the broth, plating out was done before sporulation occurred, generally about 4 days after inoculation. A loopful of the broth was streaked on plates of the following medium: glucose, 2%; yeast extract (Difco), 0.5%; peptone, 1%; agar, 2%; distilled water. To discourage bacterial growth the medium was adjusted to pH 3.5 with lactic acid. The plates were incubated at 37°. From each type of yeast colony which appeared on the plates a single one was isolated and, when necessary, purified by plating or passage through media containing antibiotics.

The isolated yeasts were classified according to the system of Lodder & Kreger-van Rij (1952). A few isolates could not be identified with species recognized by Lodder & Kreger-van Rij or with species described after the appearance of Lodder & Kreger-van Rij's monograph; some of them are described in this paper as new entities; others continue under study. In all cases, where doubts arose, our strains were compared with type strains obtained from the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Holland.

The properties of the yeasts were studied by the following methods:

Growth in liquid medium. According to Lodder & Kreger-van Rij (1952) but with the following medium: glucose, 2%; yeast extract (Difco), 0.5%; peptone, 1%; distilled water.

Appearance on streak culture. After 30 days at $\pm 25^\circ$ on the following medium: glucose, 2%; yeast extract (Difco), 0.5%; peptone, 1%; agar, 2%; distilled water.

Formation of pseudomycelium. On Dalmau plates (Wickerham, 1951) using the same medium as for the streak culture. In doubtful cases potato glucose agar (Difco) or corn meal agar (Difco) were also used.

Sporulation. According to Lodder & Kreger-van Rij (1952).

Fermentation. Glucose, galactose, sucrose, maltose and lactose fermentations according to Wickerham (1951); raffinose according to van Uden (1956).

Assimilation. According to Wickerham's (1951) technique. The following compounds were used: D-glucose, D-galactose, sucrose, maltose, lactose, raffinose, L-arabinose, D-xylose, ethanol, glycerol, D-mannitol, D-sorbitol, inositol, inulin, soluble starch, potassium nitrate.

RESULTS

One hundred and thirty-one isolates belonging to six genera and seventeen species were obtained; two isolates are described here as new species, and two others continue under study (Table 1). Findings divergent from species descriptions in Lodder & Kreger-van Rij's monograph or from descriptions of more recent species are given below. As in the published descriptions of most species the assimilation of only six or seven compounds is considered, account is given here of the assimilative properties of our bovine strains and some type strains toward the sixteen compounds we have been using (Table 2).

Table 1. *Yeasts isolated from the caeca of 252 bovines*

Species	Number of strains	Species	Number of strains
<i>Saccharomyces cerevisiae</i> Hansen	12	<i>Candida tropicalis</i> (Castellani) Berkhout	45
<i>S. chevalieri</i> Guilliermond	3	<i>C. parapsilosis</i> (Ashford) Langeron & Talice	3
<i>S. drosophilum</i> El Tabey Shehata, Mrak & Phaff	3	<i>C. krusei</i> (Castellani) Berkhout	33
<i>S. fragilis</i> Jørgensen	1	<i>C. macedoniensis</i> (Castellani & Chalmers) Berkhout	1
<i>Pichia bovis</i> n.sp.	1	<i>C. utilis</i> (Henneberg) Lodder & Kreger-van Rij	10
<i>P. membranaefaciens</i> Hansen	3	<i>C. bovina</i> n.sp.	1
<i>P. fermentans</i> Lodder	3	<i>Trichosporon cutaneum</i> (De Beurman, Gougerot & Vaucher) Ota	5
<i>Cryptococcus diffluens</i> (Zach) Lodder & Kreger-van Rij	1		
<i>Torulopsis glabrata</i> (Anderson) Lodder & de Vries	4		
<i>Torulopsis</i> sp.	2		

Saccharomyces

Twelve isolates fitted the descriptions of *Saccharomyces cerevisiae* Hansen, as given by Lodder & Kreger-van Rij (1952). L-Arabinose was used weakly by one strain, D-mannitol weakly by another strain. Three isolates were similar to *S. chevalieri* Guilliermond. According to Lodder & Kreger-van Rij (1952) *S. chevalieri* differs from *S. exiguus* Hansen, by its bigger cells and its ability to produce a pseudomycelium. As the cells of our strains are slightly smaller than the cells of the type strain of *S. chevalieri* and as they produce only a very primitive pseudomycelium, they were compared with the type strains of *S. exiguus* and of *S. chevalieri*. No differences in the fermentation and assimilation reactions were observed (Table 2). It may be concluded that *S. chevalieri* is closely related to *S. exiguus*.

Three strains were identified with *Saccharomyces drosophilum*, a species recently described by El Tabey Shehata, Mrak & Phaff (1955). Fermentation and assimilation reactions (Table 2), pigment production, type of pseudomycelium and gross morphology were the same in the bovine strains and the type strain. However, the cells of the bovine strains were smaller and measured after 48 hr. in broth $(2.6-4) \times (3.3-5.9) \mu$, whereas the cells of the type strain measure $(3.3-6.6) \times (4.6-7.3) \mu$. Furthermore, the ascospores of the

type strain are distinctly kidney-shaped, whereas the spores of the bovine strains were roundish and only rarely somewhat kidney-shaped. We do not think that these minor morphological differences justify the creation of a separate variety for the bovine strains. If, however, by the use of a larger number of compounds physiological differences should be found their separation from the species will have to be considered.

One isolate similar to *Saccharomyces fragilis* Jörgensen was found but failed to form a pseudomycelium.

Table 2. Assimilation reactions of the bovine strains and some type strains of yeasts

Strains	D-Glucose	D-Galactose	Sucrose	Maltose	Lactose	Raffinose	D-Arabinose	D-Xylose	Ethanol	Glycerol	D-Mannitol	D-Sorbitol	Inositol	Inulin	Soluble starch	KNO ₃
<i>Saccharomyces cerevisiae</i> (12)	+	+	+	+	-	+	±/-	-	-	-	±/-	-	-	-	-	-
<i>S. chevalieri</i> (3)	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>S. chevalieri</i> (t.s.)	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>S. exiguus</i> (t.s.)	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>S. drosophilum</i> (3)	+	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-
<i>S. drosophilum</i> (t.s.)	+	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-
<i>S. fragilis</i> (1)	+	+	+	-	+	+	-	+	-	+	+	+	-	+	-	-
<i>Petasospora rhodanensis</i> (t.s.)	+	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-
<i>Pichia bovis</i> (1)	+	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-
<i>P. xylosa</i> (t.s.)	+	-	+	+	-	-	-	+	+	+	+	+	-	-	-	-
<i>P. fermentans</i> (3)	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
<i>P. membranaefaciens</i> (3)	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
<i>Torulopsis glabrata</i> (4)	+	±/-	-	-	-	-	-	-	-	±/-	-	-	-	-	-	-
<i>Cryptococcus diffluens</i> (1)	+	+	+	+	+	+	+	+	+	±	+	+	+	-	+	+
<i>Candida bovina</i> (1)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. guilliermondii</i> (1)	+	+	+	+	-	+	+	+	±	+	+	+	-	+	-	-
<i>C. krusei</i> (33)	+	±/-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
<i>C. macedoniensis</i> (1)	+	+	+	-	+	+	+	-	+	+	+	-	-	+	-	-
<i>C. parapsilosis</i> (3)	+	+	+	+	-	-	±/+	+	+	±/+	+	+	-	-	+	-
<i>C. tropicalis</i> (45)	+	+	+	+	-	-	±/-	+	+	±/-	+	+	-	-	+	-
<i>C. utilis</i> (10)	+	±/-	+	±/-	-	+	-	±/-	+	+	±/-	-	-	-	-	+
<i>C. utilis</i> (t.s.)	+	-	+	+	-	+	-	±	+	+	±	-	-	+	-	+
<i>Trichosporon cutaneum</i> (5)	+	+	+	+	+	-	+	+	+	+	±/-	±/-	±/-	-	±/-	-

Figures in parentheses are the numbers of bovine strains studied. t.s.=type strain obtained from Centraalbureau or from the author of the species. +=assimilation strongly or moderately positive; ±=assimilation weakly positive; -=assimilation negative; +/-=assimilation positive in some strains, negative in other strains.

Pichia

An amended diagnosis of this genus has recently been given by Phaff (1956) to allow inclusion of species characterized by a lack of pellicle formation, by the absence or a very poor pseudomycelium or by a combination of both properties. Boidin & Abadie (1954) created the genus *Petasospora* for nitrate-negative species with hat-shaped spores, having no or weak fermentative power, and not forming an early pellicle. Phaff's definition of the genus *Pichia* allows the inclusion in the latter genus of all species so far described in *Petasospora*.

One of our bovine isolates resembles the type species of the genus *Petasospora*, *P. rhodanensis* (Ramirez & Boidin) Boidin & Abadie (1954), as well as *Pichia xylosa* Phaff, Miller & Shifrine (1956), and was compared with the type strains of both species. It was found that *Pichia xylosa* is identical with *Petasospora rhodanensis* (Table 2). As the latter species was described first,

the specific epithet *rhodanensis* has priority. As for the desirability to maintain the genus *Petasospora* we follow the suggestions of Phaff (1956) and of Kreger-van Rij (personal communication) and will transfer therefore *P. rhodanensis* to the genus *Pichia*: *P. rhodanensis* (Ramirez & Boidin) n.comb., synonym *P. xylosa* Phaff, Miller & Shifrine (1956). The bovine strain differs from this species both morphologically (short oval cells and absence of a well-developed pseudomycelium) and physiologically (assimilation of L-arabinose). It is therefore considered to belong to a new species, for which we propose the name *Pichia bovis*.

Pichia bovis sp.nov.

In medio liquido cum dextroso et peptono et extracto levedinis cellulae ovoideae, $(2.6-3.3) \times (3.3-5.3) \mu$. et $(4-5.9) \times (5.3-7.9) \mu$. aut globosae $(2-4) \mu$., singulares binae aut catenatae; sedimentum et anulus formantur. In agar peptonato cum dextroso et extracto levedinis cultura flavo-albida, mollis, nitens, glabra. Pseudomycelium nullum. Ascosporae pileiformis, 1-4 inasco. Glucosum fermentatur at non galactosum, saccharum, maltosum, lactosum, raffinolum. Assimilat dextrosolum, saccharum, maltosum, arabinosum, xylosum, alcohol aethylicum, glycerinum, mannitol, sorbitol, non vero galactosum, lactosum, raffinolum, inositol, inulinum, amidum et nitras kali.

In liquid medium after 48 hr. three types of cells are seen: small oval $(2.6-3.3) \times (3.3-5.3) \mu$.; big oval $(4-5.9) \times (5.3-7.9) \mu$.; round $(2-4) \mu$.; they are single, in pairs or in small groups (Fig. 1a); a sediment and a ring are formed. The streak culture is cream-coloured, soft and smooth with a shining surface. No pseudomycelium is formed, only a few short chains of oval cells are seen

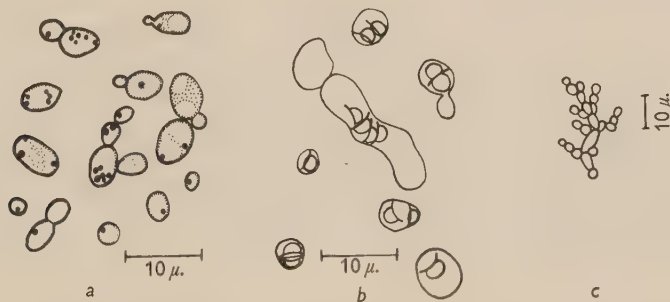


Fig. 1. *Pichia bovis*. a, organisms after 48 hr. in broth; b, asci and ascospores; c, short chains as seen in Dalmau plates. Camera lucida drawings.

(Fig. 1c). In young cultures iso- and heterogamic conjugation occurs. Asci derive from diploid cells and contain 2-4 hat-shaped spores (Fig. 1b). Glucose is fermented; galactose, sucrose, maltose, lactose and raffinose are not fermented. Assimilates glucose, sucrose, maltose, L-arabinose, D-xylose, ethanol, glycerol, mannitol, sorbitol. Not assimilated are galactose, lactose, raffinose, inositol, inulin, soluble starch and potassium nitrate.

Three sporulating, non-fermenting strains were identified with *Pichia membranaefaciens*; besides glucose and ethanol no other compounds were

utilized (Table 2). Three strains fit well in Lodder & Kreger-van Rij's description of *P. fermentans*; it is remarkable that all three assimilate D-xylose (Table 2).

Cryptococcus

One strain was identified with *Cryptococcus diffluens*. Growth at 37° was slow; many compounds were assimilated (Table 2).

Torulopsis

Four strains were identical with *Torulopsis glabrata*; besides glucose, galactose and glycerol were sometimes weakly assimilated. Two strains belonging to this genus continue under study.

Candida

Forty-five strains belonged to *Candida tropicalis*. Besides glucose, galactose, sucrose and maltose, D-xylose, ethanol, mannitol, sorbitol and soluble starch were assimilated (Table 2). The assimilation pattern of *C. parapsilosis* is almost the same; three strains were identified with the latter species. *C. krusei* was isolated thirty-three times, *C. macedoniensis* once and *C. utilis* ten times. All ten strains of *C. utilis* assimilated glucose, sucrose, raffinose, ethanol, glycerol and nitrate; the assimilation of galactose, maltose, D-xylose and mannitol was variable; none assimilated inulin whereas the type strain does. The ten bovine strains and the type strain showed the same fermentation reactions and morphology.

One strain was considered to belong to an undescribed species, for which we propose the name *Candida bovina*. As this strain produced a pseudomycelium, we had to place it in the genus *Candida*. We think, however, that *C. bovina* is closely related to the thermophilic yeast *Torulopsis pintolopesii*, from which it differs by its bigger cells, its ability to grow at room temperature, the formation of pseudomycelium and probably its habitat. We consider the generic separation of *Candida bovina* and *Torulopsis pintolopesii* a provisorium awaiting a future more natural system of classification for the yeasts now placed in the genera *Torulopsis* and *Candida*.

Candida bovina sp.nov.

In medio liquido cum dextroso et peptono et extracto levedinis cellulae ovoideae aut longovoideae $(4\text{--}5.9) \times (5.9\text{--}9.9)\mu$, binae aut singulares, sed communiter in catenis ramosis; sedimentum formatur, anulus pelliculaeque non oriuntur. In agar peptonato cum dextroso et extracto levedinis cellulae globosae $(4.6\text{--}9.9)\mu$, ovoideae $(4.6\text{--}5.9) \times (5.3\text{--}7.3)\mu$ et irregulares; cultura flavido-albida, mollis, nitens, glabra. In agar farinae maïs pseudomycelium abundat. Cellulae pseudomycelii et blastosporae longovoideae aut oblongae in catenis ramosis. Fermentat dextrosus, non vero galactosus, saccharum, maltosum, lactosum neque raffinose. Assimilat dextrosus. Galactosus, saccharum, maltosum, lactosum, raffinose, arabinosum, xylosum, alcohol aethylicum, glycerinum, mannitol, sorbitol, inositol, inulinum, amidum et nitras kalii non assimilantur.

In liquid medium after 48 hr. cells are oval and long oval, $(4\cdot5\text{--}9)\times(5\cdot9\text{--}9\cdot9)\mu$, single, in pairs, but mostly in groups (Fig. 2*a*). No ring or pellicle but a sediment is formed. On solid medium cells are round $(4\cdot6\text{--}9\cdot9)\mu$, oval $(4\cdot6\text{--}5\cdot9)\times(5\cdot3\text{--}7\cdot3)\mu$, and irregular (Fig. 2*b*). The streak culture is cream-coloured, soft and smooth with a shining surface. On corn meal agar pseudomycelium formation is abundant. It consists of dense, much ramified chains of oval and cylindrical cells (Fig. 2*c*). Only glucose is fermented and assimilated. No assimilation of galactose, sucrose, maltose, lactose, raffinose, arabinose, xylose, ethanol, glycerol, mannitol, sorbitol, inositol, inulin, soluble starch and potassium nitrate.

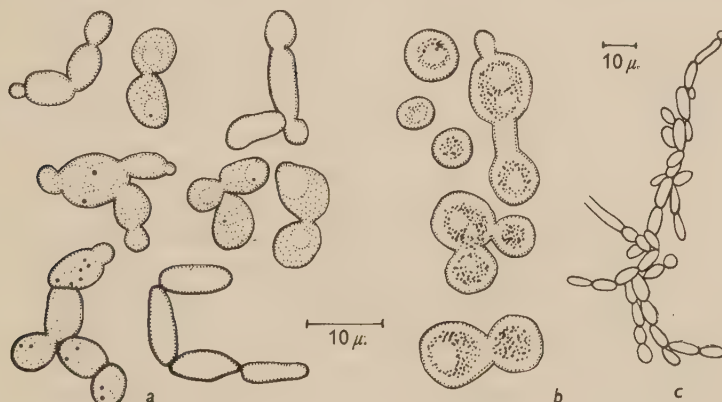


Fig. 2. *Candida bovina*. *a*, organisms after 48 hr. in broth; *b*, after 12 days on solid medium; *c*, pseudomycelium as seen in Dalmau plates with corn meal agar. Camera lucida drawings.

Trichosporon

Five strains were identified with *Trichosporon cutaneum* as described by Lodder & Kreger-van Rij (1952). Two strains used mannitol, sorbitol and inositol but not soluble starch; the three other strains assimilated soluble starch but did not use mannitol, sorbitol or inositol. The type strain obtained from the Centraalbureau has become pleomorphic and results of assimilation reactions were uncertain. It is felt that the species *T. cutaneum* is heterogenic and further research on this subject is needed.

DISCUSSION

The most important single fact among our results seems to be the apparent absence of *Candida albicans* from the bovine intestinal tract. *C. albicans* belongs to a group of yeasts which may be called obligate saprophytes of warm-blooded animals having its normal habitat in the digestive tract of man and various other animals. Although normally a harmless saprophyte, this yeast may cause under special conditions disease of man, birds and other animals ('moniliasis'). According to different authors 14–30·8% of humans harbour *Candida albicans* in their faeces (Benham & Hopkins, 1933; Schnoor, cited by Yo Bwan Hie, 1954; Lawler *et al.*, cited by Yo Bwan Hie, 1954; Marples &

Di Menna, 1952). Of 378 fowls studied by Jordan (1953) 21 % harboured *C. albicans* in the crop. *C. albicans* has been isolated from turkeys suffering from moniliasis (Blaxland & Fincham, 1950). According to Skinner (1947) *C. albicans* has been recorded in laboratory animals, rats, pigeons and the European hedgehog. Coutelen & Cochet (1942) reported the isolation of *C. albicans* from rabbit, guinea-pig and rat; Talice (1932) referred to its isolation from fowl, a lamb, a cat, a dog, a turkey and donkeys. Only exceptionally can *C. albicans* be isolated from non-animal sources and there is no evidence that *C. albicans* has a natural habitat outside the human and animal body (Negroni & Fisher, 1941; Di Menna, 1955; van Uden, Matos Faia & Assis-Lopes, 1956). It would seem from our results, however, that the bovine intestinal tract does not constitute a natural habitat for *C. albicans*.

Torulopsis glabrata, another obligate saprophyte of warm-blooded animals, was found four times in our series. The potential pathogenicity of this species is very small; histopathological pictures suggestive of histoplasmosis may be produced in experimental infections of rats and mice (Lopez Fernandes, 1952). All ten strains studied by Lodder & Kreger-van Rij (1952) were of human origin; numerous strains were isolated by ourselves from human faeces and from the vagina and once we isolated a strain from the liver of an elephant. The only known isolation of *T. glabrata* outside the human or animal body was the one of Phaff, Mrak & Williams (1952) who isolated a strain from the surface of iced shrimps.

Candida bovina is also most probably an obligate saprophyte of warm-blooded animals. *Torulopsis pintolopesii*, to which it seems nearly related, is highly adapted to life in warm-blooded animals and does not grow at room temperature; it was originally isolated from white mice (van Uden, 1952). Since then it has been isolated from mice, rats, pigeons and meadow voles (Artagaveytia-Allende, 1953; Parle [personal communication of Di Menna]; Aschner, Halevy & Awram, 1954; Emmons, personal communication). Further research with a more exhaustive isolation technique is needed to verify the incidence of *Candida bovina*. This yeast is somewhat fastidious and may easily be overgrown by more vigorous species during isolation procedures.

A second group of yeasts, which may be called facultative saprophytes of warm-blooded animals, is composed of yeasts which live naturally both inside and outside the body; *Candida tropicalis* and *C. krusei* belong to this group. Both species are frequently encountered and have been isolated from man and other animals as well as from materials such as beer, brewery yeast, dough, fruit, kefir, fodder yeast, tea fungus (Lodder & Kreger-van Rij, 1952). It would seem from our results that both *C. tropicalis* and *C. krusei* are dominant species in the bovine intestinal yeast flora. Of the two, only *C. tropicalis* is a potential pathogen; it is less virulent than *C. albicans* (Mackinnon, 1936). *Trichosporon cutaneum* belongs to the same group, together with *Candida parapsilosis*, *C. guilliermondii* and *C. macedoniensis*. With the possible exception of some strains of *Trichosporon cutaneum* none of them has pathogenic properties.

The incidence of *Candida utilis* (10 strains) is relatively high. This species is

used for the manufacture of fodder yeast in many countries (White, 1954), but to the best of our knowledge no fodder yeast of this kind is used in Portugal. It may well be that *C. utilis* is not a simple intestinal passer-by introduced with food but a facultative saprophyte of bovines and probably of other warm-blooded animals. The fact that two of Lodder & Kreger-van Rij's strains were isolated from human sputum supports this view.

Before something can be said of the ecological behaviour of *Pichia bovis* more strains will have to be isolated.

The other species isolated from our bovine series probably belong to a third group of yeasts which do not live saprophytically in the digestive tract but come principally from ingested food. Ecologically well-known yeasts, e.g. *Saccharomyces cerevisiae* and *S. chevalieri*, may be placed without reserve into this group. Also *Cryptococcus diffluens* surely is not an intestinal saprophyte as it thrives very badly at 37°. With other species, e.g. *Pichia membranaefaciens* and *P. fermentans*, some doubt may arise about their ecological classification.

From the veterinary point of view our results throw some light on bovine yeast diseases. Thrush or moniliasis of the mouth and intestine seems to be rare in bovines. According to Skinner (1947) it was stated by Hagan: 'Some authors refer to monilia infections of the oral mucosa in calves and colts. No additional information about them is available. Presumably they are of little consequence.' Our findings that the common cause of moniliasis, *Candida albicans*, is substituted in the bovine intestinal yeast flora by the much less virulent *C. tropicalis* might partly explain the apparent rareness of thrush in bovines. Yeast infections of the udder, however, seem to be more frequent. A severe form is caused by *Cryptococcus neoformans* and epizootics of this type have been reported by Pounden, Amberson & Jaeger (1952) and by Simon, Nichols & Morse (1953). So far *C. neoformans* has not been found as a saprophyte in the human or animal digestive tract, but has been isolated from soil (Emmons, 1951; Ajello, 1956), from pigeon nests and dung (Emmons, 1955) and from a *Dentocrotonus* beetle (Shifrine & Phaff, 1956). In our series of 252 bovines no strain of *C. neoformans* was isolated. It would seem therefore that *C. neoformans* mastitis is an exogenous mycosis. The same cannot be said of the other form of yeast mastitis which normally is fairly mild, occurs sporadically or in small epizootics and is caused by various yeast species. Cases have been reported by Rolle (1934), Murphy & Drake (1947), Lernau, Shapiro & Aschner (1947), Stuart (1951) Burkey, Burkner, Underwood & Swett (1951). From these cases not fully-identified yeasts of the genera *Trichosporon* and *Pichia* have been isolated. Stuart (1951) isolated a *Candida* sp. different from *C. albicans*; Murphy & Drake (1947) reproduced the disorder experimentally with a strain of *Trichosporon* sp. isolated from a case of mastitis. So far *Candida albicans* does not seem to have been isolated from cases of yeast mastitis; this may be related to the apparent absence of *C. albicans* from the bovine intestinal tract. On the other hand, since we isolated from the bovine intestinal tract species of the genus *Candida* other than *C. albicans*, as well as species of *Pichia* and *Trichosporon*, the possibility must be kept in mind that infection in yeast

mastitis, not caused by *Cryptococcus neoformans*, may originate from faecal material.

Cultures of *Pichia bovis* n.sp. and *Candida bovina* n.sp. have been deposited with the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Netherlands.

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The Applicability of the Hypothesis of Independent Action to Fatal Infections in Mice given *Salmonella typhimurium* by Mouth

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SUMMARY: Mice were challenged by mouth with a suspension containing equal numbers of streptomycin-sensitive (Str^-) and streptomycin-resistant (Str^+) variants of *Salmonella typhimurium*. These variants were of equal virulence but the Str^+ variant grew more slowly *in vivo* than the Str^- variant. The LD 50 dose contained *c.* 5×10^5 bacteria. Heart blood obtained from mice dying from many LD 50 doses nearly always contained a great excess of the Str^- variant, but blood from mice dying from less than one LD 50 dose contained either Str^- , Str^+ , or a mixture of Str^- and Str^+ variants. The appearance of the Str^+ variant alone in the latter mice strongly suggests that these fatal infections were initiated by a very small number of organisms or possibly by a single organism. It is therefore concluded that these organisms were acting independently. In this system, it is likely that any bacterium which enters the tissues from the gut can initiate a fatal infection and that the probability of effecting such an entrance almost entirely determines the probability of an inoculated bacterium causing a fatal infection.

The inoculation of a partially resistant host with many bacteria will often cause a fatal infection when the inoculation of one bacterium is very unlikely to do so. Of several hypotheses advanced to account for this phenomenon, the most satisfactory appears to be that described elsewhere (Meynell & Stocker, 1957) as the 'hypothesis of independent action' which postulates (*a*) that bacteria act independently after inoculation, and (*b*) a mean probability ($1 > p > 0$) per inoculated bacterium of initiating a fatal infection which is constant and unaffected by the number of bacteria inoculated. On this hypothesis therefore, there will always be a chance that the death of a host inoculated with many bacteria will be caused by the progeny of only one of the inoculated bacteria. Although many of the inoculated bacteria may multiply to some extent, the total toxic effect of their clones will never be fatal and will be negligible compared to the toxic effect of the clone descended from only one of the inoculated bacteria. The progenitor of this clone may thus be said to have initiated the fatal infection.

A well-known alternative hypothesis is that of the minimal lethal dose which postulates that if the dose exceeds a critical minimum size, death is inevitable; while if it is smaller than the minimum, the host is certain to survive. Thus, a lethal dose might be supposed to saturate the host defences so that death follows the multiplication either of all the inoculated bacteria or of those bacteria not needed for saturation. In the latter case, a fatal infection could

* Present address.

be initiated by one organism on the rare occasion when the inoculated dose exceeded the minimal lethal dose by precisely one organism. This event is clearly very unlikely. Hence, if the hypothesis of the minimal lethal dose is valid, many bacteria will initiate each fatal infection as a result of their co-operative action on the host.

The following test should distinguish these hypotheses (Kunkel, 1934). A suspension is prepared which contains equal proportions of recognizable variants of the chosen pathogen, each variant being distinguished only by a marker character unrelated to its virulence. A series of challenge doses is prepared from this suspension in which the most potent dose contains many LD₅₀ doses and the least potent, only a fraction of one LD₅₀ dose. These doses are administered to groups of hosts. Any hosts which die are sampled to determine the composition of the bacterial population responsible for death. Any host in which a fatal infection was initiated by one bacterium should contain a great preponderance, if not a pure culture, of only one variant. It can be shown (Meynell & Stocker, 1957) that where the hypothesis of independent action is applicable, most fatal infections following the inoculation of one or less than one LD₅₀ dose are initiated by a single organism if the hosts do not differ in resistance. When the hosts vary in resistance, as is probably always the case, one can only say that the chance that a given death was due to the multiplication of one bacterium is inversely proportional to the size of the dose. It follows that if the hypothesis of independent action is valid, most hosts dying after challenge by small or large doses should yield respectively either one variant, alone or predominantly, or all the variants. But if the hypothesis of the minimal lethal dose is valid, all fatal infections are initiated by many bacteria and hence each fatally infected host should yield all the variants.

The LD₅₀ dose of *Salmonella typhimurium*, when given by mouth to mice, is 5×10^5 organisms. The possibility that the hypothesis of independent action applied to this system has been tested by giving mice by mouth a mixture of streptomycin-sensitive (Str⁻) and streptomycin-resistant (Str⁺) variants of this organism. The composition of the bacterial population responsible for death was determined by examination of samples of heart blood removed post mortem. The predictions have to be modified for this system for although, as will be shown, both variants are about equal in virulence, the Str⁻ variant grows more rapidly than the Str⁺ variant so that any mouse in which a fatal infection is initiated by both variants will probably yield at least a preponderance of the Str⁻ variant post mortem.

The results indicate that the inoculated bacteria act independently, not co-operatively. Other evidence suggests that nearly every organism which passes from the gut to the tissues is capable of initiating a fatal infection. The experiment with mixed inocula therefore also shows that the bacteria are independent during their passage from the gut lumen.

METHODS

Bacteria. A streptomycin-sensitive strain of *Salmonella typhimurium*, designated GLeB, was isolated from a sick mouse and passaged several times by intraperitoneal injection. After the last passage, a streptomycin-resistant variant was isolated by inoculating plates of nutrient agar containing 200 μ g. streptomycin/ml. with a heavy suspension of the passaged strain. Both strains were preserved by freeze-drying. The Str⁺ strain, also used by Meynell (1955), has the same efficiency of plating on streptomycin agar as on nutrient agar.

Challenge doses were prepared by mixing equal volumes of three overnight broth cultures of each variant inoculated from the dried stock cultures. The mixture was diluted as necessary in quarter-strength Ringer's solution.

Inoculations. All mice were kept without food, water or sawdust for 18 hr. before challenge by mouth: those challenged by intraperitoneal injection were kept under normal conditions throughout. In the first experiment, each mouse was infected by mouth by placing 0.2 ml. suspension in the end of a drinking tube suspended from the cage lid. This was usually taken within 20 min. In the other experiments each mouse was given 0.2 ml. of suspension through a polythene catheter passed beyond the mid-point of the oesophagus.

Mice. Webster BSVS mice were used in Exp. 1 and for the titrations of the Str⁻ variant, and were kept in individual pots. The other experiments were performed on the stock mice of the Postgraduate Medical School which were kept in large glass pots in groups of 10.

Examination of the post mortem heart blood. Heart blood was removed from each mouse post mortem and added to 5 ml. digest broth which was thereafter stored at 4°. Portions of the diluted blood were plated by loop in a standard manner on nutrient agar and on streptomycin agar (200 μ g./ml. agar). Control experiments showed that when the Str⁺ variant formed less than 10 % of the total population, the number of colonies on streptomycin agar was much less than on nutrient agar. When the Str⁺ variant formed more than 10 % of the population, separate colonies were scored by subculture from nutrient agar to streptomycin agar.

RESULTS

The response of mice to challenge by the Str⁻ variant

When groups of mice were challenged by mouth with serial tenfold dilutions of the Str⁻ variant, the proportion of mice dying never increased steadily with increase in dosage and inversions in the sequence were always present (Table 1). The slope of the dose-response curve (estimated graphically on a log-dose/probit-mortality plot) was about 0.7, which was markedly less than the maximum slope of 2.00 predicted for infective systems by the hypothesis of independent action (Meynell & Stocker, 1957). This observation showed that the resistances of individual mice differed considerably and that it was therefore impossible to predict exactly either the mean probability of an inoculated organism causing a fatal infection or the probability that a given fatal infec-

tion was initiated by one bacterium. The former probability was clearly small since the LD 50 dose of the Str⁻ variant when given by mouth was *c.* 5×10^5 bacteria. It will be shown below that the variants are approximately equal in virulence in Expts. 1 and 3 and probably also in Expt. 2. Mice always responded far more uniformly to the Str⁻ variant when given by intraperitoneal injection (Table 1); the LD 50 dose was *c.* 2.5 organisms by this route.

Table 1. *Response of mice to challenge by the Str⁻ variant of Salmonella typhimurium*

		Challenge by mouth				
		<i>d</i> =	10 ³	10 ⁴	10 ⁵	10 ⁶
Titration:	(1) P =		0/7	3/7	1/7	4/7
	(2)		1/7	0/7	1/7	2/7
	(3)		1/7	1/7	1/7	6/7

LD 50 = 5×10^5 bacteria approximately.

		Challenge by intraperitoneal injection				
		<i>d</i> =	5×10^{-2}	5×10^{-1}	5×10^0	5×10^1
		P =	0/10	1/10	5/10	9/10

LD 50 = 2.5 organisms approximately.

d = mean number of viable bacteria/dose.

P = number of mice dying/number of mice challenged.

The proportions of the Str⁻ and Str⁺ variants present in heart blood obtained post mortem from mice challenged with a mixture of both variants

Expt. 1. The results are shown in Table 2 which gives the dose and route of inoculation for each group of mice; the proportion of mice dying; the proportion of each variant present in the challenge dose and in the heart blood samples with the number of colonies scored; and the number of days each mouse survived after challenge. It can be seen that the heart blood of all mice dying after challenge by many LD 50 doses, given either by mouth or by intraperitoneal injection, contained a great preponderance of the Str⁻ variant. These observations clearly showed that the Str⁻ variant outstripped the Str⁺ variant *in vivo*. Therefore, if every fatal infection resulted from the multiplication of a number of bacteria drawn at random from the challenge dose, and never from one bacterium as postulated by the hypothesis of independent action, it would be expected that the Str⁻ variant would always predominate in the heart post mortem. The third part of Table 2 shows that this result was not obtained since 3/8 mice dying from a dose of 10^4 bacteria (0.02 LD 50 dose) yielded only the Str⁺ variant. Two other mice yielded a mixture containing a preponderance of the Str⁻ variant, such as would be expected if either one or many bacteria of each kind initiated these fatal infections. Three mice yielded only the Str⁻ variant; their deaths may have been due to Str⁻ bacteria only but alternatively, any contributing Str⁺ bacteria might have been outgrown and might not have entered the heart blood by the time death occurred.

Expt. 2. This experiment was designed to show that the appearance of the Str⁺ variant alone in 3/8 mice dying from 0.02 LD 50 dose in Expt. 1 was not due to selection of Str⁺ organisms of greater virulence or growth rate than the

Table 2. *The proportions of Str⁻ and Str⁺ variants of Salmonella typhimurium present in the post mortem heart blood of mice challenged by both variants (Expt. 1)*

Route of inoculation	Dose and mortality	Sample	Proportion of each variant		No. colonies scored	Survival period (days)
			Str ⁻ variant	Str ⁺ variant		
Oral	10 ⁷ organisms (20 LD 50 doses); 5/5 died	Challenge dose	0.57	0.43	180	—
		Post mortem heart blood	0.997	0.003	8 × 10 ³	6
			0.999	0.001	10 ⁴	5
			0.975	0.025	600	9
			0.896	0.104	73	6
			0.999	0.001	10 ⁴	8
Intra-peritoneal	2.5 × 10 ⁴ organisms (10 ⁴ LD 50 doses) 5/5 died	Post mortem heart blood	0.993	0.007	5.3 × 10 ³	6
			0.983	0.017	460	7
			0.959	0.041	197	5
			0.836	0.164	116	5
			0.984	0.016	181	6
Oral	10 ⁴ organisms (0.02 LD 50 doses) 10/48 died; 8 scored	Post mortem heart blood	0	1.0	310	15
			0	1.0	78	21
			0	1.0	27	15
			1.0	0	10 ⁷	—
			1.0	0	4.4 × 10 ⁴	10
			1.0	0	3 × 10 ³	11
			0.993	0.007	10 ³	7
			0.99	0.01	1.5 × 10 ³	10

original Str⁻ variant. The challenge dose was therefore prepared from the Str⁺ present alone in one of the mice in Expt. 1 and the stock strain of the Str⁻ variant. The proportion of the latter in the dose was 0.59 (98 colonies scored). Four out of sixteen mice and 5/60 mice died from doses of 5 × 10⁷ organisms (20 LD 50 doses) and 5 × 10⁴ organisms (0.1 LD 50 dose), respectively. The heart blood samples obtained post mortem from all save one of these mice contained the Str⁻ variant either alone or with a very small proportion of the Str⁺ variant (<0.01); the proportion of the Str⁻ variant in the exceptional sample was 0.2. More than 10⁴ colonies were scored from each mouse.

This result strongly suggested that the predominance of the Str⁺ variant in 3/8 mice dying in Expt. 1 was not a consequence of mutation to greater virulence or growth rate. So few mice died from the smaller dose that the absence of mice yielding the Str⁺ variant alone is without significance.

Expt. 3. The results are given in Table 3 and resemble those of Expt. 1. One mouse, possibly of unusually high resistance, yielded the Str⁺ variant alone after challenge by 1.25 × 10⁸ bacteria (250 LD 50 doses), the other eleven mice in this group all yielding the Str⁻ variant either alone or in a marked excess. Twenty-one mice died from a dose of 1.25 × 10⁵ bacteria (0.25 LD 50 dose); the Str⁺ variant was recovered alone from 4/16 mice examined. Ten of the remaining mice yielded only the Str⁻ variant and two yielded both variants.

Expt. 4. The challenge dose was prepared from a strain of the Str⁺ variant isolated in pure culture from a mouse in Expt. 3, mixed with the stock strain of the Str⁻ variant in the ratio 0.425/0.575. Eight out of twenty and 48/120 mice

died from doses of 5×10^7 bacteria (100 LD₅₀ doses) and 5×10^4 bacteria (0.1 LD₅₀ dose), respectively. All mice yielded the Str⁻ variant alone, Str⁺ organisms were not isolated from any mice.

Table 3. *The proportions of Str⁻ and Str⁺ variants of Salmonella typhimurium present in the post mortem heart blood of mice challenged by mouth by both variants (Expt. 3)*

Dose and mortality	Sample	Proportion of each variant		No. colonies scored	Survival period (days)
		Str ⁻ variant	Str ⁺ variant		
—	Challenge dose	0.66	0.34	76	—
1.25 × 10 ⁸ organisms (250 LD ₅₀ doses) 17/19 died; 12 scored	Post mortem heart blood	0	1.0	148	9
		1.0	0	10 ² –10 ³	6
		1.0	0	33	8
		1.0	0	c. 10 ³	8
		1.0	0	c. 10 ²	9
		1.0	0	c. 10 ³	9
		1.0	0	c. 10 ⁵	9
		0.92	0.08	80	5
		0.94	0.06	34	5
		0.96	0.04	81	6
		0.96	0.05	27	8
		0.9	0.1	c. 10 ²	8
1.25 × 10 ⁵ organisms (0.25 LD ₅₀ doses) 21/140 died; 16 scored	Post mortem heart blood	0	1.0	177	6
		0	1.0	217	9
		0	1.0	101	13
		0	1.0	90	13
		1.0	0	c. 10 ²	5
		1.0	0	c. 10 ²	6
		1.0	0	36	6
		1.0	0	24	7
		1.0	0	c. 10 ³	8
		1.0	0	c. 10 ³	9
		1.0	0	c. 10 ²	9
		1.0	0	10 ² –10 ³	9
		1.0	0	c. 10 ³	9
		1.0	0	c. 10 ³	11
		0.96	0.04	45	9
		0.83	0.17	71	6

Other experiments. In earlier experiments, mice were challenged by mouth with 10⁴ bacteria (0.02 LD₅₀ dose) of a suspension containing a proportion of 0.64 of the Str⁻ variant. Four days later the mice were killed and the bacterial population of the mesenteric glands examined. The Str⁺ variant was isolated alone from two glands, the Str⁻ variant alone being isolated from the other ten glands which yielded salmonellas.

DISCUSSION

Fatal infections following the inoculation of many LD₅₀ doses of a mixture of equally virulent Str⁻ and Str⁺ variants of *Salmonella typhimurium* are assumed to be initiated by both Str⁻ and Str⁺ bacteria, whichever hypothesis

is valid. Nevertheless, the post-mortem heart blood of all, save one, of the mice dying after challenge by such doses contained an excess, if not a pure culture, of the Str⁻ variant. It may therefore be concluded that when a fatal infection is initiated by a mixture of Str⁻ and Str⁺ bacteria, as would always be the case if the hypothesis of the minimal lethal dose were generally valid, Str⁻ bacteria should predominate in the heart blood at post mortem.

Many mice which died from less than one LD₅₀ dose yielded only the Str⁺ variant. Hence, the bacteria which initiated the fatal infection in these mice must all have been Str⁺ and their number must have been quite small (say, less than 10), or else at least one Str⁻ bacterium would have been included and its progeny would have been present in the heart blood post mortem. It seems implausible to suggest that a fatal infection can only be initiated by the co-operation of such a small number of bacteria, so that it seems justifiable to conclude that it could have been initiated by only one bacterium. This implies that the bacteria were acting independently as postulated by the hypothesis of independent action, which therefore applies to this system. As indicated in the introduction, this result is very unlikely to be obtained if the hypothesis of the minimal lethal dose be valid.

The proportion of one variant in the challenge dose in Expts. 1 and 3 was approximately equal to the proportion of mice which yielded a pure culture of that variant following challenge by less than one LD₅₀ dose, showing that the mean probability of an inoculated bacterium causing a fatal infection was about the same for each variant (i.e. that the variants were probably equal in virulence). This conclusion could have been further tested by determining the LD₅₀ dose for each variant separately, but titrations using moderate numbers of mice would have detected only extreme differences in virulence because of the presence of gross variation in host resistances (Table 1).

Since both variants were about equal in virulence, the Str⁻ variant must have outstripped the Str⁺ *in vivo*, just as it can be shown to do *in vitro*, owing to its more rapid rate of growth. It is therefore not surprising that mice whose deaths were due to Str⁺ bacteria only, survived longer than those which yielded Str⁻ bacteria only (Tables 2 and 3). The difference in survival times may have been greater than the tabulated results suggest, for Expts. 1 and 3 were ended after 3 and 2 weeks, respectively, and some late deaths may have been missed.

Expt. 4 differed from the previous experiments in that no fatally infected mice yielded any Str⁺ bacteria in their heart blood. It is unlikely that the virulence or the growth rates of the variants had altered owing to genotypic changes and other experiments suggest that such alterations may be phenotypic in origin. For example, on one occasion, the strains used in Expt. 1 were mixed in equal parts and 10⁵ LD₅₀ doses inoculated by intraperitoneal injection. At post mortem, an unusually high proportion (*c.* 0.3) of the bacteria in the heart blood were Str⁺. Alterations of this sort might invalidate experiments intended to show that mutation to greater virulence, etc., was not responsible for the occurrence of the Str⁺ variant alone in the heart blood of some mice. However, the validity of Expt. 2 is unaffected, since mice challenged by many LD₅₀ doses responded in the same way as those in Expts. 1 and 3.

The question then arises as to why only a few, or possibly only one, of the inoculated organisms were able to multiply to a fatal extent when the rest could not. It will be noted that the experiment with mixed inocula does not provide an answer, for it can only estimate the number of bacteria which initiate each fatal infection. The following three experiments suggest that in this system the bacteria initiating fatal infections may have been the only bacteria which passed from the gut to the tissues where they were of maximal virulence: (a) the resistance of mice to oral infection by the Str⁺ variant is greatly lowered when the normal gut flora is previously altered by giving streptomycin by mouth (Bohnhoff, Drake & Miller, 1954; Meynell, 1955). The LD 50 dose of the Str⁺ variant of *Salmonella typhimurium* was decreased in this way from $c. 5 \times 10^5$ to 1.2 organisms. Unless streptomycin affects the state of the gut secretions, this observation shows that the small probability of an inoculated organism causing a fatal infection is not due to most of the dose being killed within the gut lumen; (b) Meynell & Stocker (1957) inoculated mice with a mixture of two variants of *S. typhimurium* by intraperitoneal injection. The post mortem heart blood did not usually contain only one variant after administration of 0.2 LD 50 dose (200 bacteria), as predicted by the hypothesis of independent action. Instead, only a marked predominance of one variant or the other was found. (The presence of the lesser proportion was ascribed to the effects of a terminal breakdown in resistance which allowed bacteria to multiply which otherwise would have been restrained by the host defences.) Since many pure infections by the slow-growing Str⁺ variant were observed after challenge by mouth, it seems likely that the number of bacteria which entered the tissues from the gut was less than 200, the number inoculated in the earlier experiments; (c) the entry of one bacterium might always be fatal in this system, for the Str⁻ variant is very virulent by intraperitoneal injection (Table 1). However, this argument may be misleading since the virulence of *S. typhimurium* is greatly affected by the route of inoculation (Dutton, 1955).

The present experiments resemble those performed by Liu & Henle (1953) with a mixture of influenza A and B viruses inoculated into the allantoic cavity of the chick embryo, a system to which either hypothesis might conceivably apply since the ID 50 dose contains $c. 10$ virus particles (Donald & Isaacs, 1954). When 10^3 ID 50 doses or less are inoculated, influenza B grows more slowly than influenza A (Liu & Henle, 1951) so that these viruses can be likened to the Str⁺ and Str⁻ variants respectively. Eggs receiving 10^3 ID 50 doses (Liu & Henle, 1951) or 32 ID 50 doses (Liu & Henle, 1953) of a mixture containing equal numbers of ID 50 doses of each virus therefore yielded an excess of influenza A after incubation. Yet eggs inoculated with <16 ID 50 doses sometimes yielded an excess of influenza B with a small proportion of influenza A. This finding, like the occurrence of mice yielding only the Str⁺ variant, is most simply explained on the assumption that inoculated particles act independently in the early stages of infection. The presence of the small proportion of influenza A in the eggs containing a majority of influenza B may be ascribed to a breakdown in resistance caused by the growth of influenza B, that is, to co-operation occurring in the later stages of infection. Some eggs

yielded influenza B alone; this finding is not strong evidence of independence when the ID50 dose contains so few particles, because the inocula these eggs received may, by chance, have contained only influenza B.

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A Preliminary Study of Tobacco Mosaic Virus by the Gel Diffusion Precipitin Tests

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SUMMARY: A number of proteins can be separated from purified preparations of tobacco mosaic virus; they differ from each other antigenically and all differ from the intact virus by not possessing all antigenic determinant groups possessed by the virus. Some of these proteins are easily detached from the virus by such mild treatments as placing it in a protein solution or in agar gel. These are antigenically identical with the 'X-protein' which remains in the supernatant fluid when the virus is sedimented by ultracentrifugation from sap of infected plants. More of these proteins are detached from the virus by placing it in borate buffer at *c.* pH 8.7. When a more drastic treatment is applied, such as incubation at *c.* pH 10, which disintegrates a proportion of the virus, still more of these proteins are released, but then some proteins antigenically different from those of 'X-protein' are also released.

Different variants of the gel diffusion precipitin test have been applied to preparations of tobacco mosaic virus (TMV) by Sang & Sobey (1954), van Slogteren (1955), Jeener, Lemoine & Lavand' Homme (1954) and Commoner & Rodenberg (1955). All except Commoner & Rodenberg found two or three antigenically different components, and Jeener *et al.* gave evidence that the major component is identical with the virus and the others with the non-infective protein which appears in the plant as a result of infection with TMV and remains in the supernatant fluid when the virus is sedimented from fresh plant extracts by ultracentrifugation. This protein will be called X-protein, although the name was originally given by Takahashi & Ishii (1952, 1953) to a component with a rate of electrophoretic mobility slightly smaller than half that of the virus, and subsequently more components with different mobilities were found (Commoner, Yamada, Rodenberg, Wang & Basler, 1953; Jeener *et al.* 1954). X-protein is precipitated by antisera made against normal preparations of TMV. Some workers found that the antisera absorbed by X-protein still precipitate TMV (Jeener *et al.* 1954; Starlinger, 1955) and other workers that they do not (Bawden & Pirie, 1956).

The minor components could be distinguished in the gel diffusion tests from the major component because they diffused through agar much faster than did the major component and thus had relatively small particles. Preparations of TMV, homogeneous in the gel diffusion test, should, therefore, be obtained if they are prepared by ultracentrifugation, or, if prepared by other methods, should be made homogeneous by ultracentrifugation. Preliminary experiments showed, however, that these expectations were not realized and that purified preparations of TMV normally contain serologically distinguishable components which are not separable by ultracentrifugation. Experiments were, therefore, made to gain some information about their origin and relationship

to *X*-protein, and also to the similar protein that can be split from purified preparations of TMV by incubation at pH 9.5–10.5 (Schramm, 1943). This protein was called *A*-protein by Schramm, Schumacher & Zillig (1955) and considered to be identical with *X*-protein by Schramm & Zillig (1955) and by Starlinger (1955) who found that antiserum to TMV absorbed with *A* protein did not precipitate *X*-protein, although it still precipitated TMV. Schramm & Zillig (1955) obtained electrophoretically homogeneous preparations of *X*-protein with the same rate of mobility as *A*-protein (slightly smaller than half the rate of TMV), and suggested that the various components with different mobilities observed by other workers resulted from differences in the degree and type of aggregation of one basic material. The results of the present work show that *X*- and *A*-proteins differ antigenically from intact typical virus, but are not identical, and each is antigenically heterogeneous.

METHODS

Antisera were prepared by intravenous injection into rabbits of virus preparations purified without the use of an ultracentrifuge. Usually about 10 mg. virus were injected intravenously into rabbits six times at weekly intervals and the animals bled about 10 days after the last injection. Sometimes the animals were kept after such a series of injections for several months, given a second series of two or three injections and then bled.

Tobacco mosaic virus (TMV) was purified, usually from sap of systemically infected tobacco plants, by repeated alternate precipitation by one-third saturation with $(\text{NH}_4)_2\text{SO}_4$ and by adjusting the pH value to 3.4. The procedure was continued as long as it was obvious that contaminating material was being removed with the supernatant fluid when precipitated virus was sedimented by centrifugation (*c.* 10,000 rev./min.), or centrifuged down after the virus had been dissolved. Tomato bushy stunt virus (BSV) was purified from sap of infected tomato plants by several successive precipitations by one-third saturation with $(\text{NH}_4)_2\text{SO}_4$, removal of some contaminating material that became insoluble at pH 3.4, and crystallizing the virus by adding enough $(\text{NH}_4)_2\text{SO}_4$ to produce faint opalescence at room temperature and then keeping the preparation for a few weeks at 2°. The purified preparations of both viruses were finally dialysed against distilled water and stored at 2°. Usually after dialysis it was possible to remove more contaminating material by centrifugation. The final preparations of the viruses were almost colourless at *c.* 4% (w/v) concentrations.

Two variants of the gel diffusion precipitin test were used; one was done in test tubes of 1.1 cm. diameter (the 'tube method'), and the other in Petri dishes of 9–10 cm. diameter (the 'Petri dish method'). In both 0.5% (w/v) agar in 0.9% (w/v) NaCl solution (with addition of sodium azide in the proportion of 1/5000) was used.

The tube method was a modification of that originally used by Oakley & Fulthorpe (1953): 1 ml. of antiserum diluted in agar was poured first into the tube; when this had solidified 1 ml. of agar alone was poured in and when this

had solidified 1 ml. of antigen diluted in agar was poured in. The Petri dish method was a modification of the original method of Ouchterlony (1949). Twenty ml. agar were poured into a Petri dish. Holes, usually rectangular, were made in the agar either by placing pieces of metal in the dish before pouring agar and removing them after the agar solidified, or by removing pieces of solidified agar with a scalpel and spatula. The holes were filled with antisera and with antigens, both diluted in agar. The dilution factor was never greater than 1/10, so that the antiserum and antigen solutions in agar solidified to about the same degree of firmness as did the surrounding agar. (For diluting antisera and antigens the agar was melted and cooled to 42°.)

Diffusion of antigens and antibodies from their original positions through the intervening agar towards each other results in differently directed concentration gradients and precipitates form within the agar where two conditions are fulfilled: the ratio of concentrations of antigen and antibody is such that precipitation can occur, and the total concentration of either is above some limiting value. The term 'lines' of precipitate will be used, although really the precipitate occupied disk-shaped spaces in the tubes and spaces of various shapes in the plates. The lines usually started to be visible within a few days, and then grew in intensity and thickness, sometimes moving their positions. Observation of the lines was continued for a month or longer. The terms 'layer' of antigen or antiserum (for the tube method) and 'area' of antigen or antiserum (for the Petri dish method) will be used to describe the original positions of antigens and antisera, although antigens and antibodies may have diffused and no longer have been confined to these positions.

The formation of several lines has three possible explanations. (1) There may be several different antigens and several different antibodies, and each antigen-antibody system may establish the ratio suitable for precipitation in a different position. The antigens may or may not possess common determinant groups, but must differ from each other by possession of some groups which are not possessed by the others, because only antibodies that do not correspond to any determinant group of a given antigen can pass unhampered beyond the position where that antigen has formed a precipitation line, to form a precipitation line elsewhere with another antigen. (2) Homogeneous antigen may produce something similar to the Liesegang phenomenon, resulting in a multiplicity of lines, rather close to each other (this has been discussed by Wilson & Pringle, 1954). (3) Several lines may be formed by an antigenically homogeneous material because it exists in a number of forms differing widely from each other in physical properties (e.g. particle size, diffusion rate, solubility). This is particularly relevant to virus preparations such as those of TMV, which do contain various particles differing from each other in this way.

When two lines cross without much affecting each other's course, as they may do in agar plates when two or more antigenic preparations are tested against one or more antisera, the conclusion that the two lines are formed by two different antigens seems unavoidable. On the other hand, when two lines, formed by two materials present in two different preparations, bend towards each other and fuse to form a perfect continuation of each other, the conclusion

is that the two materials are antigenically identical. When, however, the lines remain separate and do not cross (they are always parallel when the tube method is used), the possibilities (2) and (3) cannot be ruled out. Thus the appearance of several lines which did not cross was considered only as an indication, but not as sufficient evidence that there were as many different antigens in a tested material. Attempts were then made to find other evidence and to separate and identify the antigens.

RESULTS

Multiplicity of precipitation lines obtained with preparations of TMV and BSV, purified without the use of ultracentrifuge

Fig. 1 shows a typical result obtained by the tube method with the preparation of TMV and the antiserum mostly used in this work. There are three lines of precipitation, one (*a*) near the antigen layer, and the other two (*b* and *c*) close to each other about half way between antigen and antiserum layers. A very similar result was obtained with a preparation of BSV and its antiserum. All the lines were specific for each virus preparation, for they were formed only with homologous antisera even when the two viruses were propagated on the same host (tomato). It is possible, therefore, that the virus preparations contained at least three different antigens, which may, or may not, possess common determinant groups.

The photograph shown in Fig. 1 was taken 6 days after the test was set up; later the lines became more intense and thicker. The increase in thickness occurred upwards (i.e. towards the antigen layer), the position of the lower edges remaining almost unaltered. Eventually the two lower lines (*b* and *c*) coalesced, and the upper line (*a*) became the most intense and the thickest and it encroached into the antigen layer.

The position of (the lower edges of) the lines depends on the ratio of initial concentrations of antigen and antiserum at their starting positions. Table 1 shows that when the concentrations of the two materials vary but the ratio is kept constant, the positions of the lines do not alter appreciably. When the ratio is altered, the lines form farther away from the initial position of the material whose concentration is increased in relation to the other material.

Table 1. *The dependence of the position of precipitation lines on the ratios of initial concentrations of antigen and antiserum*

The figures show approximate distances (in mm.) between the lower edge of the line *c* (see Fig. 1) and the interface between the antigen layer and the blank agar layer. (The height of the blank layer was about 10 mm.)

Dilution of antiserum in the bottom layer of agar	Initial concentrations of TMV in the top layer of agar		
	0.1 %	0.025 %	0.006 %
	Distance of line <i>c</i> (mm.)		
1/10	3	2	1
1/40	5	3	2
1/160	No visible line	5	2.75

The three lines of precipitation shown in Fig. 1 were not obtained with all the different antisera used. All formed the line *a* (nearest the antigen layer) and this was the only line formed by some, whereas the other antisera formed another line further away from the antigen layer, or another two lines (*b* and *c*).

Usually rabbits given only few injections of small amounts of TMV produced antisera which formed only one line (*a*), whereas further injections of the same animals with the same TMV preparation resulted in antisera which formed, in addition to the line *a*, another line or another two lines (*b* and *c*). Sang & Sobey (1954), who used another method of the gel diffusion test, also noticed that only one precipitation line was obtained with antisera produced by injecting rabbits twice with small amounts of TMV, and that a second line appeared after a more prolonged course of injections. This is the first evidence that all the three lines were not formed by an antigenically homogeneous material, for the most likely explanation of these results is that the rabbits which received small amounts of a TMV preparation, produced antibodies that reacted only with the antigen which formed the line *a*, whereas after more injections with larger amounts of antigen, antibodies were also produced which reacted with the antigen or antigens that formed the other two lines.

Separation of antigens by absorption with antiserum

If different antigens and antibodies are involved in forming different precipitation lines, it should be possible to remove some of the antigens and leave the others by precipitation with an antiserum which contains only some of the antibodies. To test this two antisera were used: antiserum I, produced by the usual series of injections and which formed the three lines shown in Fig. 1;

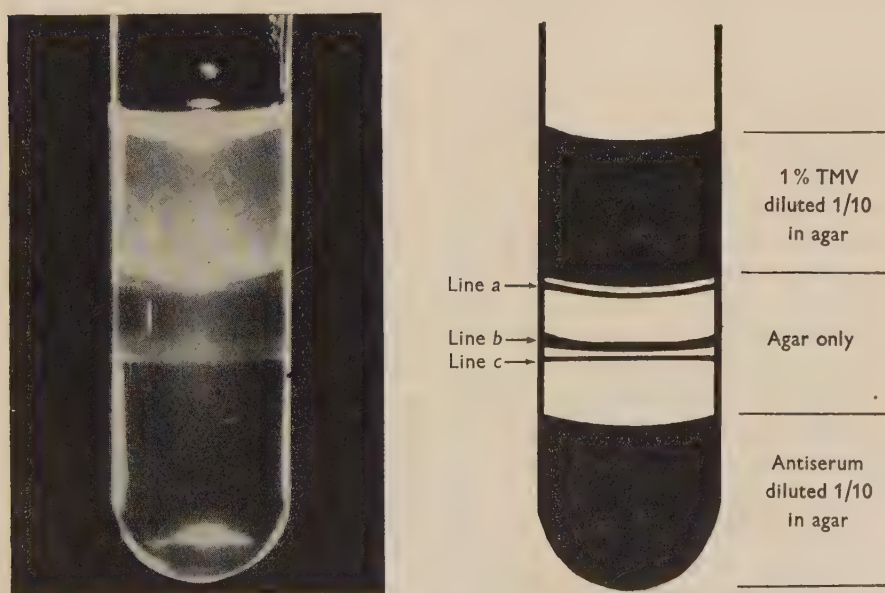


Fig. 1. Precipitation lines formed by a TMV preparation with an antiserum.

antiserum II, produced by a single injection of 2 mg. TMV and which formed only one line (*a*). The correspondence of the single line formed by antiserum II with the line *a* formed by antiserum I was confirmed by the Petri dish method, when the two lines joined, forming a continuation of each other (Fig. 2).

The virus preparation was absorbed with antiserum II. The details are given in Table 2, which shows that when the ratio of the amount of the antiserum to that of the virus was as in tube no. 3, or higher, all the material that could precipitate with antiserum II was precipitated, whereas some other material that could precipitate only with antiserum I still remained in solution. Typical virus particles were precipitated by antiserum II, for nothing could be seen when the supernatant fluid from tube no. 3 after the first test (Table 2) was examined in the electron microscope, and no lesions were obtained when the fluid (undiluted, and diluted 1/10 and 1/100) was inoculated to leaves of *Nicotiana glutinosa*.

Table 2. *Absorption of a TMV preparation with antiserum II*

The second test was made by adding 0.1 ml. of antiserum II or I at a dilution of 1/10 to 2 ml. of each supernatant fluid obtained by removing the precipitates formed during the first test by centrifugation for 10 min. at 10,000 rev./min. All fluids contained 0.9% NaCl, and during the tests the tubes were incubated for 3 hr. at 50° and then overnight at room temperature. + signs indicate the presence and the degree of precipitation; - signs indicate the absence of precipitation.

Tube no.					
1	2	3	4	5	6
First test: 3 ml. of 0.02% solution of TMV + 3 ml. of antiserum II at a dilution of:					
1/10	1/20	1/40	1/80	1/160	Saline
++++	++++	++++	++++	+++	-
Second test with antiserum II					
-	-	-	±	++	++++
Second test with antiserum I					
+	+	+	++	+++	++++

As it was possible that the negative results of the electron-microscopic examination and of the infectivity test were obtained because too low a concentration of the material remained in the supernatant fluid of the tube no. 3, another experiment was made in which antiserum II and the virus preparation were mixed in the same proportions but 40 times more concentrated. The precipitate was removed by centrifugation. Nothing could be found in the supernatant fluid by examination with the electron microscope. The material that remained in the supernatant fluid was then freed as much as possible from serum proteins. This was done by one-fourth saturation with $(\text{NH}_4)_2\text{SO}_4$, re-solution of the precipitate in water, precipitation by adjusting to pH 3.5 and re-solution in water by adjusting to pH 7. This procedure was repeated and the final volume of the resulting solution was made up to half that of the original TMV + antiserum mixture. The solution precipitated up to a dilution of 1/64 with antiserum I (used at 1/200), but it did not precipitate with antiserum II.

Lesions were not produced on leaves of *Nicotiana glutinosa* after inoculation with the solution (undiluted and diluted 1/10 and 1/100).

The results of the absorption experiment showed that the virus preparation contained at least two serologically different materials: the characteristic virus particles and a small amount of some material with particles too small to be observed with the electron microscope, and which were non-infective. Antiserum I contained antibodies to both materials, whereas antiserum II contained antibodies to the typical virus particles only. The line *a* in Figs. 1 and 2 was formed by the virus and the lines *b* and *c* by the other material. As this material formed two lines, presumably it contained at least two serologically different components.

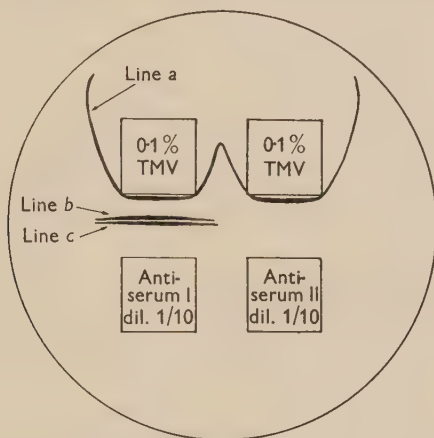


Fig. 2. A comparison of two different antisera to TMV.

Separation of antigens by ultracentrifugation

If the lines *b* and *c* of Figs. 1 and 2 are indeed formed by antigenic materials with relatively small particles, it should be possible to separate these materials from the virus by ultracentrifugation and thus obtain a serologically homogeneous virus preparation. Yet sedimentation from 1% solutions of TMV in water, or in 0.9% NaCl, at pH values round 7 gave no such separation. The redissolved pellet, obtained after two successive sedimentations at 40,000 rev./min. (80,000 *g*) for 30 min., behaved in the gel diffusion precipitin tests in exactly the same manner as the original virus preparation and the supernatant fluid produced no lines. By contrast with TMV, serologically homogeneous preparations of BSV were immediately obtained by two successive sedimentations by high-speed centrifugation, under the same conditions.

The preparation of TMV in M/15 phosphate buffer (pH 7) appeared electrophoretically homogeneous when examined in a Perkin-Elmer electrophoresis apparatus, even when the virus concentration was as high as 2%. However, when tested in 0.2M-borate buffer (pH 8.7), the preparation appeared to contain two other components besides the virus. The mobility of one was slightly smaller than half that of the virus, and that of the other only slightly less than that of the virus. The two components still formed the two electrophoretic

peaks different from that of the virus, after the pH value had been brought back to 7 by dialysis first against water and then against M/15 phosphate buffer (pH 7). The mobilities of the two components exactly corresponded to those found by Jeener *et al.* (1954) for components of X-protein. Judging from the areas under the electrophoretic peaks, the total content of the two components of the virus preparation would be about 2–5 % of the total protein. It looks, therefore, as if some materials that were combined with the virus at *c.* pH 7, split from the virus in the borate buffer at pH 8·7 and did not recombine when the pH value was brought back to 7. It seemed possible that these materials are the same as those which gave the lines *b* and *c* in the gel diffusion precipitin tests; therefore another attempt was made to separate the components of the virus preparation by high-speed centrifugation. This time 1 % (w/v) virus solution in borate buffer (pH 8·7) was kept overnight at 2° and then centrifuged for 30 min. at 40,000 rev./min. (80,000 *g*). This did result in a separation. The pellet (which was dissolved in the original volume of water and the pH value adjusted to 7) appeared serologically homogeneous, forming only the line *a* in the gel diffusion tests, whereas the supernatant fluid (which was dialysed against 0·9 % NaCl, when the pH value fell to about 7) formed only the lines *b* and *c*.

The infectivity of the material in the pellet, measured by the local lesion method on *Nicotiana glutinosa*, was about equal to that of TMV which had not been incubated at pH 8·7. The material that formed the line *a* will, therefore, be called 'virus', and the symbol TMV will be used for standard virus preparations. The material contained in the supernatant fluid was non-infective (no lesions when *N. glutinosa* was inoculated at 300 mg./l.). The ultraviolet absorption spectrum had a maximum at *c.* 275 m μ . and a minimum at 255 m μ . Thus, if the material contained any nucleic acid at all, it was much less than in TMV. Electron-microscopic examination of the material after precipitation by 0·4 saturation with (NH₄)₂SO₄ and re-solution, showed rods similar to those of TMV and also numerous disks with holes near the centre, similar to those found by Schramm & Zillig (1955) in partially aggregated preparations of A-protein. The material resembles, therefore, both A- and X-proteins in its ability to aggregate into large rod-shaped particles.

The fact that the material could not be separated from the virus by ultracentrifugation or by electrophoresis before the preparation had been incubated in borate buffer at pH 8·7, seems incompatible with the fact that the material formed precipitation lines *b* and *c*, separate from the line *a* formed by the virus, in the gel diffusion tests at *c.* pH 7, and remained in solution when TMV was precipitated by a suitable antiserum, also at *c.* pH 7. This apparent anomaly might be explained by assuming that when the virus is adsorbed to a colloid such as agar gel or serum protein, some of the material, which forms the lines *b* and *c*, is dislocated from the virus particles. To test the assumption, a solution containing 1 % (w/v) of TMV and 1 % of rabbit serum albumin at pH 7, was centrifuged for 30 min. at 80,000 *g*, and the gel diffusion precipitin test set up with the pellet (redissolved in the original volume of water) and with the supernatant fluid. The pellet formed the lines *a*, *b* and *c*, whereas the super-

natant fluid formed only the lines *b* and *c*. Thus some of the material which formed these lines did separate from the virus in the presence of 1% serum albumin.

Comparison with A- and X-proteins

As various points of similarity between the material which gives *b* and *c* lines in the gel diffusion precipitin tests, and the A- and X-proteins were noticed, it seemed possible that the material might be identical with one of them or with both, if indeed they are identical as suggested by Schramm & Zillig (1955) and by Starlinger (1955). To test this, A- and X-proteins were prepared, and each was compared with a standard preparation of TMV by the gel diffusion precipitin test.

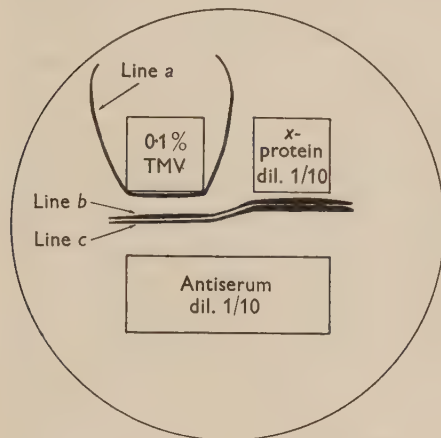


Fig. 3. A comparison of X-protein with a TMV preparation.

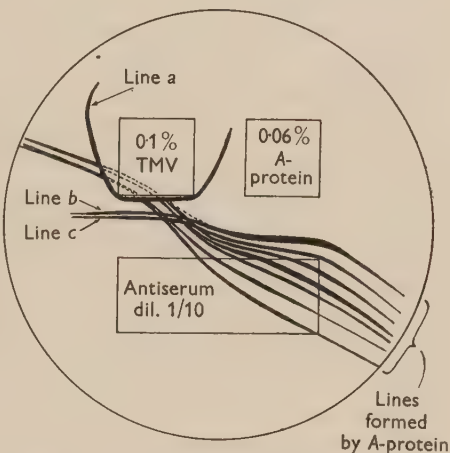


Fig. 4. A comparison of A-protein with a TMV preparation.

X-protein was prepared by removing TMV from fresh sap of infected tobacco plants by high-speed centrifugation and concentrating the X-protein by precipitation from the supernatant fluid by 0.4 saturation with $(\text{NH}_4)_2\text{SO}_4$ and re-solution in a volume of water equal to one-tenth the original volume of sap. The fluid was dialysed against water and clarified by removing insoluble material by low-speed centrifugation (6000 g).

Figure 3 shows the result of a comparison of X-protein with the standard preparation of TMV when both were tested on the same agar plate against the antiserum to TMV. The TMV preparation formed its usual three precipitation lines (*a*, *b* and *c*), whereas X-protein formed two rather ill-defined lines which deviated from their course to fuse with the lines *b* and *c* of the TMV preparation and to form their continuations. X-protein did not form any line which corresponded to the line *a* of the TMV preparation. Thus X-protein seems to be antigenically identical with the material or materials which formed the lines *b* and *c*, and does not seem to contain anything identical with the material which formed line *a*.

A-protein was prepared from the virus which was previously separated by

ultracentrifugation at pH 8.7 from the material that formed the lines *b* and *c*. A 1% solution of the virus was adjusted to pH 10.2 with 0.1N-NaOH and kept for 24 hr. at 2°. The solution was then dialysed against M/15 phosphate (pH 7) for 24 hr. at 2° and examined in the electrophoresis apparatus. Three peaks were observed, similar to those obtained by Schramm *et al.* (1955): one small peak with a mobility about 1.7 times that of TMV, another large peak corresponding to unchanged TMV, and a third large peak corresponding to *A*-protein, with a mobility just less than half of that of TMV. The area under the third (*A*-protein) peak was about half of that under the second (TMV) peak.

Some of the component which formed the third peak (*A*-protein) was removed from the electrophoresis cell without the other components. The preparation of *A*-protein thus obtained gave a UV absorption spectrum with a maximum between 275 and 280 m μ , and a minimum at about 252 m μ . Thus, if this protein contained nucleic acid at all, it was much less than in TMV. The preparation produced only a few lesions per half-leaf of *Nicotiana glutinosa* when inoculated at a concentration of 300 mg./l., in contrast to many on the opposite halves of the leaves inoculated with TMV at 5 mg./l. The few lesions probably occurred because the *A*-protein was contaminated with infective virus in the electrophoretic cell. On the other hand, infectivity of the virus which remained infective in the TMV preparation after incubation at pH 10.2, appeared even greater than that of the original virus preparation. The increased infectivity of virus particles which remained infective after exposure to alkali made impossible any estimation of the proportion of particles whose infectivity was destroyed by this exposure.

Figure 4 compares *A*-protein with the standard preparation of TMV when both were tested on the same agar plate against the antiserum to TMV. The TMV preparation again formed the usual three precipitation lines (*a*, *b* and *c*), whereas the *A*-protein formed at least seven lines. On the left of the original position of *A*-protein the lines were so close to each other that most of them merged, but they fanned out and became separate farther to the right, i.e. towards the space between the original positions of *A*-protein and of antiserum, and most of the lines passed across the area of the original position of the antiserum. The lines which were the farthest from the antigen area first formed much nearer to that area than shown in Fig. 4, and then moved farther away across the antiserum area.

The course of the lines formed by *A*-protein was altered by the presence of the TMV preparation. The course they would have taken had the TMV preparation not been there, is shown by broken lines (this is copied from a plate identical to that shown in Fig. 4 except that it did not contain the TMV preparation). The lines *b* and *c* crossed several lines formed by *A*-protein, to fuse with two of them which were nearest to the antigen area. These lines deviated from their course to cross the other lines and form continuations of lines *b* and *c*. It is concluded, therefore, that *A*-protein contains two antigenically different components, one of which is antigenically identical with the material forming the lines *b* and *c*, and thus antigenically identical with

X-protein. The multiplicity of lines formed by the two components suggests that each was a mixture of antigenically different materials.

The lines that were formed by *A*-protein which did not deviate to fuse with the lines *b* and *c* continued their independent course until they encountered line *a*. They did not continue beyond line *a*, but they appeared to the left of the line *a* after it turned sharply upwards (i.e. away from the antiserum area). In other words, no line could be formed by *A*-protein at any point which lay on the side of the *a* line opposite to that of the antiserum. It seems, therefore, that none of the antibodies which could precipitate components of *A*-protein, could pass unhampered through the line *a*. As the line *a* was formed by the typical virus particles, and as antibodies can diffuse freely through precipitates formed in agar by unrelated antigen-antibody systems (Wilson & Pringle, 1955), the conclusion reached is that the antiserum did not contain any antibodies specific for *A*-protein which were not also specific for typical virus particles.

DISCUSSION

There is no conclusive evidence that the proteins which can be separated from purified preparations of TMV, and which are antigenically identical with *X*-protein, are identical with *X*-protein in all respects. *X*-protein is found in sap of infected tobacco plants and can be separated from virus by high-speed centrifugation. It might be concluded that *X*-protein and the virus existed in the sap separately, i.e. were not combined with each other. However, the protein which is antigenically identical with *X*-protein, seems to be combined with the virus with different degrees of firmness. Some can be detached by such mild treatment as placing in a protein solution or in agar gel, more can be split off by a somewhat more drastic treatment, such as placing in borate buffer at pH 8.7. Still more can be released by a still more drastic treatment, such as exposure to alkali at *c.* pH 10, but then some of the virus particles disintegrate, and another kind of protein, different antigenically from *X*-protein, is also released. There is even no conclusive evidence that all the successive yields of protein antigenically identical with *X*-protein, are identical in all respects among themselves. Since, however, they share with *X*-protein a number of other common features (e.g. electrophoretic mobility, particle size, ability to aggregate into particles resembling those of the virus) it seems very probable that they are identical with *X*-protein. If this be assumed, the material which is released from the virus at pH 10, consists of *X*-protein and of another antigenically different protein which it is proposed to designate as *Y*-protein. The protein designated by Schramm *et al.* (1955) as *A*-protein is not, therefore, identical with *X*-protein, but is a mixture of *X*- and *Y*-proteins.

Both *X*-protein and *Y*-protein seem to consist of mixtures of antigenically different materials. *X*-protein formed two precipitation lines with one of the antisera to TMV, and *Y*-protein formed at least five other lines. Multiplicity of lines cannot alone be considered as sufficient evidence for a corresponding multiplicity of antigenically different components, but there is corroborative evidence for this with *X*-protein, namely that some antisera formed only one

line with it. If the two lines were not a result of the presence of two different antigens, and if some sort of mechanism operated whereby an antigenically homogeneous material produced multiple lines (as discussed in Methods), the multiple lines would be expected with all antisera of comparable strength. It can be assumed, therefore, that there are at least two antigenically different components in X-protein. Antisera which formed only one line did not necessarily lack antibody to one of these components, but may have contained different kinds of antibodies in such a ratio that the two lines coincided, for positions of lines depend on the relative concentrations of antigens and antibodies at the initial positions. Y-protein probably consists of at least five antigenically different components, but there is no corroborative evidence for this. It seems, therefore, that the protein units of molecular weight of about 90,000 into which, according to Schramm & Zillig (1955), TMV is disintegrated by alkali treatment, or those of molecular weight of about 20,000 which according to an X-ray study by Franklin & Holmes (1956) are arranged helically along the virus particle, are not all identical, but are of several kinds, differing from each other antigenically.

It does not seem likely that the presence of free X-protein in the sap of infected plants is a result of disintegration of the virus, because then one would expect free Y-protein also to be present. The possibility that free X protein is a precursor of the virus is also made less likely by the absence of free Y-protein. As some X-protein is easily detached from the virus, it is quite possible that free X-protein was originally attached to the virus, although it is also possible that it was originally free and subsequently got loosely adsorbed to the virus.

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The Water and Solid Content of Living Bacterial Spores and Vegetative Cells as Indicated by Refractive Index Measurements

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SUMMARY: Refractive index measurements were made on the spores and vegetative cells of strains of *Bacillus cereus*, *B. cereus* var. *mycoides* and *B. megaterium* by phase contrast and interference microscopy with protein immersion. The refractive indices of the spores were found to be very high and comparable with that of dehydrated protein, suggesting that they contained very little water. Those of the vegetative cells were much lower, and indicated a solid content of about 30 %, w/v.

Because of their relatively high resistance to heat and other lethal agents, it has long been thought probable that bacterial spores contain appreciably less water than the vegetative forms. Only a few attempts to measure the water content of spores or vegetative cells have, however, been described (Virtanen & Pulkki, 1933; Henry & Friedman, 1937), and the methods used, all of which involved bulk weighing and varying degrees of desiccation, were necessarily rather crude and imprecise.

Refractive index measurements can now be made on microscopical objects with considerable accuracy (e.g. Barer, Ross & Tkaczyk, 1953; Mitchison & Swann, 1953; Davies, Wilkins, Chayen & La Cour, 1954; Ross, 1954; Barer & Joseph, 1954), and, as was first pointed out by Davies & Wilkins (1952) and by Barer (1952*a*), there is a close correlation between the refractive index of a cell and its water and solid content. It has been shown that the majority of substances found in living cells have approximately the same specific refraction increment (α) of *c.* 0.0018. This means that their combined refractive indices increase by about 0.0018 for every 1 % rise in their w/v concentration (Davies *et al.* 1954; Barer & Joseph, 1954).

In the present work, the refractive indices of the vegetative cells and spores of three selected organisms were measured with a view to obtaining a more accurate indication of their water and solid content than hitherto.

MATERIAL AND METHODS

Cultures. The vegetative cells and spores measured were of single typical strains of *Bacillus cereus*, *B. cereus* var. *mycoides*, both isolated from milk, and *B. megaterium* (National Collection of Type Cultures 2607). All were grown on a 1 % Lemco 1 % peptone agar medium and incubated at 30°.

Method of measuring the vegetative cells. The refractive indices of the vegetative cells from 18 hr. cultures were measured by the method of immersion refractometry first used by Barer & Ross in 1952. The cells were suspended in a range of concentrations of protein in aqueous solution (bovine plasma albumin, fraction V, supplied by the Armour Laboratories, Eastbourne), of measured refractive index, and examined with a phase-contrast microscope. They then all appeared darker than the background when mounted in solutions having a lower refractive index than their own, and brighter in solutions of higher refractive index. In media with refractive indices approximating closely to that of the cells, they became almost invisible. This method is a very sensitive one, capable of detecting refractive index differences of 0.001, which is approximately equivalent to 0.5 % of cell solids. It was found that the refractive indices of the individual vegetative cells varied by greater amounts than this, so that over a limited range of concentrations (in no case exceeding a refractive index range of 0.009, or 5 % of cell solids), dark and bright cells were both visible. The solution in which bright and dark cells were found to occur in approximately equal numbers was the one whose refractive index was taken as being equivalent to the mean refractive index of the cell population.

Method of measuring the spores. The method for the vegetative cells could not be used for the spores since it was found that their refractive indices were all higher than the most concentrated protein solutions obtainable, and non-toxic media of higher refractive index are difficult to find. Nevertheless, the fact that the middle of the spores all actually appeared bright under the phase-contrast microscope when they were mounted in water (Pl. 1, fig. 1), together with their small size, did indicate that the spores must have a considerably higher refractive index than the vegetative cells; because, with the particular phase plate used, this meant that the light passing through their maximum thickness was retarded by at least a quarter of a wavelength (Barer, 1952*b*). It is not possible, however, actually to measure such retardations with a phase-contrast microscope.

Instead, an interference microscope was used to measure the retardations of light through the spores when mounted in two media of different refractive indices, and from these measurements their mean refractive index was calculated. A Smith interference microscope (manufactured by Messrs Charles Baker of Holborn) was used, and measurements were made with a 2 mm. double focus objective in green light provided by a tungsten 'pointolite' lamp in conjunction with an Ilford 807 gelatin filter. The transmission of the filter with the light from this microscope lamp was investigated with a spectroscope, since these gelatin filters often show slight individual variations from the maker's specifications. It was found to give a narrow green band with a maximum intensity at 542 m μ ., which was taken as being the mean wavelength of the light used.

The maximum retardation in phase of the light through the middle of each spore was measured by extinction point measurement, first in preparations mounted in distilled water (refractive index 1.334), (Pl. 1, figs. 2, 3), and then in concentrated aqueous protein solutions (which had varying refractive indices

of about 1.40). The phase retardations of ten different spores were thus measured (as angles) in each of the two media, and the mean of each of the two sets of measurements taken.

The mean spore thickness was then calculated from the formula

$$t = \frac{\phi_1 - \phi_2}{(n_2 - n_1) 360} \times \lambda, \quad (1)$$

where t = the mean spore thickness (along the optical axis of the microscope) in μ ., ϕ_1 = the mean phase retardation of the spores in water, ϕ_2 = the mean retardation of the spores in the protein medium, n_1 = the refractive index of water (1.334), n_2 = the refractive index of the protein medium, and λ = the mean wavelength of the light used in μ . (0.542). The mean refractive index of the spores, n_s , was then calculated from the formula

$$n_s = \left(\frac{\phi_1}{360} \times \frac{\lambda}{t} \right) + n_1. \quad (2)$$

This method was similar to that employed by Barer in 1953, except that the phase-change measurements, instead of being made on the same individual cells suspended successively in two media, were made on a different sample of spores in each medium. This was because of the experimental difficulty of completely replacing one mounting medium with another without washing all the spores out of the field.

RESULTS

Vegetative cells. The refractive indices of the individual vegetative cells of *Bacillus cereus* were all found to lie between 1.3845 and 1.3880 and they had a mean refractive index of 1.3865. The other two organisms were rather more variable; those of *B. cereus* var. *mycoides* lay between 1.3960 and 1.4030 with a mean of 1.4000, and those of *B. megaterium* between 1.3830 and 1.3920 with a mean of 1.3880.

Spores. The results of two sets of measurements on different cultures of the spores of each organism are shown in Table 1. It will be seen that in each case the values for the mean thickness of the spores and their mean refractive index varied very little in the two successive experiments. The values for ϕ_1 are all comparable since the mounting medium was water in every case, but the values for ϕ_2 are not, because the refractive indices of the protein mounting media were different in the two experiments.

The accuracy of the values obtained. As has already been mentioned, the direct immersion and matching method used for measuring the refractive indices of the vegetative cells is very critical, and accurate to the nearest 0.001 which is less than the variation found in the refractive indices of the individual cells in each culture.

With the method used for the spores, it was not possible to obtain values for the refractive index of individual spores since, necessarily, different spores had to be measured in each medium. In deducing their mean refractive index, it

was assumed that the mean phase change and thickness of the spore population behaved in essentially the same way as that of an individual spore.

Such an assumption would seem to be a reasonable one to make in the absence of evidence to the contrary, but circumstances can be envisaged where it might give rise to a certain degree of error in the values obtained for the mean refractive index of the population. This might happen, for instance, if the refractive indices of individual spores varied appreciably according to their thickness, as might occur in a population containing germinating spores, which are known to swell and imbibe water; but the material used contained only resting spores.

Table 1. *The mean angular retardations of light, of wavelength 542 mμ., through spores mounted in water and in protein solutions measured by the interference microscope and their mean thicknesses and refractive indices calculated therefrom*

	<i>B. cereus</i>		<i>B. cereus</i> var. <i>mycoides</i>		<i>B. megaterium</i>	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Refractive index of protein medium (n_2)	1.3925	1.3960	1.3925	1.4000	1.3925	1.3880
Mean retardation in water (degrees) (from 10 measurements of different spores) (ϕ_1)	126.8	132.8	123.6	124.6	134.0	130.2
Mean retardation in protein (degrees) (from 10 measurements of different spores) (ϕ_2)	84.8	87.4	86.4	79.4	95.0	96.0
Mean thickness in $\mu.$ (t)	1.08	1.10	0.96	1.02	1.00	0.95
Mean refractive index (n_s)	1.521	1.513	1.528	1.519	1.537	1.540

It was thought advisable to get additional evidence to support this assumption, and so an alternative method was also used to obtain approximate values for the refractive indices of individual spores in the case of *Bacillus cereus*. Spores of this organism were mounted in distilled water and the phase retardations through ten spores were measured in the same way as before, but the thickness of each spore was also measured as accurately as possible with an eyepiece micrometer. The refractive index of each spore, n_s , was then calculated from the formula

$$n_s = \left(\frac{\phi_1}{360} \times \frac{\lambda}{t} \right) + n_m, \quad (3)$$

where ϕ_1 = the phase retardation through each spore, λ = the mean wavelength of the light used (0.542 $\mu.$), n_m = the refractive index of mounting medium (1.3340) and t = the thickness of each spore estimated to the nearest 0.2 $\mu.$ These measurements, and the refractive indices calculated from them, are shown in Table 2.

This method, however, is not a very critical one, and its accuracy is limited by the accuracy with which one can measure the spore thickness by direct measurement. With the 2 mm. double focus objective used, it is very doubtful

if the thicknesses were measured accurately to more than the nearest 0.4μ , and the final column of the table shows the limits of the refractive index of each spore assuming a maximum error of $\pm 0.2\mu$ in their measured thicknesses.

Table 2. *Individual measurements of the maximum width and angular retardation of light of wavelength 542 m μ . through spores of B. cereus mounted in water, and the refractive indices of the spores calculated therefrom*

Spore no.	Width (to the nearest 0.2μ .) measured by micrometer eyepiece (t) (μ .)	Retardation through spore in water (degrees) (ϕ_1)	Refractive index of spore (n_s)	Range of refractive index of spore, assuming a max. error in width (t) measurement of $\pm 0.2\mu$.
1	0.9	96	1.495	1.466–1.542
2	0.9	114	1.524	1.490–1.579
3	0.9	112	1.521	1.488–1.576
4	1.1	128	1.509	1.482–1.549
5	1.1	120	1.490	1.474–1.534
6	1.1	124	1.504	1.478–1.541
7	1.1	126	1.507	1.480–1.544
8	1.1	134	1.519	1.490–1.558
9	1.3	150	1.510	1.485–1.539
10	1.5	176	1.511	1.490–1.538

(Range of refractive index of vegetative cells of *B. cereus* 1.384–1.388.)

In spite of the relative inaccuracy of the method, however, two important conclusions can be drawn from these results.

(1) There appears to be no appreciable correlation between the refractive index and the thickness of the spores; and the fact that the phase change increases with the spore thickness suggests that the refractive indices of both the larger and smaller spores is similar. (The same kind of correlation between phase change and thickness was found in the cultures of *Bacillus cereus* var. *mycoides* and *B. megaterium*.)

(2) Even allowing for the relative inaccuracy of the method, it will be seen that the refractive indices of the spores are nevertheless all considerably greater than those of the vegetative cells.

It is also interesting to see that the average of these ten phase-change measurements is 128.0° as compared with the values of 126.8 and 132.8° obtained previously, and the average of their refractive indices is 1.509 compared to the previous values of 1.512 and 1.513 (see Table 1). While too much should not be made of this, it does serve to indicate that the values obtained by the previous method are probably, in general, correct.

A further indication of the accuracy of the double-immersion method is given by the results obtained by using exactly the same technique to measure the mean refractive index of the vegetative cells of *Lactobacillus bulgaricus* (Ross, 1957, to be published). Here, the values obtained could be compared with those obtained by direct immersion refractometry, and it was found that, in nine separate cultures, these differed from each other and from those obtained by direct immersion and matching by a maximum of 0.0085 , which is equivalent to less than 5% of cell solids.

CONCLUSIONS AND DISCUSSION

Water content. If one assumes that the mean refraction increment (α) of the dissolved substances in the vegetative cells is 0.0018 per 1 % rise in concentration, the mean w/v concentration of cell solids, C , can then be found from the formula (Barer, 1956):

$$C = \frac{n_c - 1.334}{0.0018}, \quad (4)$$

where n_c = the mean refractive index of the vegetative cells. Table 3 shows the percentage of cell solids thus calculated. It also shows the percentage of water, assuming, first that the solids in the vegetative cells are composed predominantly of protein, and secondly that 1 g. of protein occupies 0.75 ml. in aqueous solution, which is approximately true.

Table 3. *Solid and water content of vegetative cells, spores and other materials calculated from refractive index measurements*

Material	Mean refractive index	Assuming $\alpha = 0.0018$		Assuming $\alpha = 0.0015$
		Mean solid content (in g./100 ml.)	Mean water content (in g./100 ml.)	Mean water content (in g./100 ml.)
Vegetative cells				
<i>B. cereus</i>	1.386	29.0	78.0	—
<i>B. cereus</i> var. <i>mycoides</i>	1.400	36.5	72.5	—
<i>B. megaterium</i>	1.388	30.0	77.5	—
Spores				
<i>B. cereus</i> , Expt. 1	1.512	99.0	26.0	11.0
Expt. 2	1.513	99.5	25.5	10.5
<i>B. cereus</i> var. <i>mycoides</i>				
Expt. 1	1.528	108.0	18.0	3.0
Expt. 2	1.519	102.5	23.0	7.5
<i>B. megaterium</i> , Expt. 1	1.537	113.0	15.0	-1.5
Expt. 2	1.540	114.5	14.0	-2.5
Leather	1.530*	109.0	18.0	1.5
Wool	1.540*	114.5	14.0	-2.5
Dried casein	1.540*	114.5	14.0	-2.5

* Chamot & Mason (1938).

The exact interpretation, in terms of water and solid content, of the much higher values obtained for the refractive indices of the spores is more controversial, since it has been found that the results obtained from the extrapolation of formula (4) above do not accord exactly with the experimental evidence available (Davies *et al.* 1954; Barer & Joseph, 1954; Barer, 1956). This suggests that the refractive index of dried protein may be rather lower than might be expected, and Davies *et al.* (1954) have suggested that the refraction increment (α) of dried protein may be as low as 0.0015. It is certain, however, that these values indicate a very high content of solids, since they are comparable with the values obtained experimentally for the refractive indices of almost completely dehydrated protein products such as leather (1.53), wool and dried

casein (both 1.54), quoted by Chamot & Mason (1938), and they strongly suggest that the spores must contain very little water. Table 3 also shows the solid and water concentrations of the spores calculated on the same assumption used in the case of the vegetative cells. It is quite possible, however, that the values for the water content thus calculated are rather too high, and the final column of the table shows the even lower values obtained assuming the refraction increment of 0.0015 suggested by Davies *et al.* 1954. This method is, of course, only capable of indicating the total water content of the spores. It is not capable of showing whether, as was suggested by Friedman & Henry in 1938, a proportion of this occurs as 'bound water'.

Previous measurements of the water content of vegetative cells and spores. Henry & Friedman (1937) estimated the water content of the spores and vegetative cells of two of the three organisms used in the present investigation from bulk weighing and drying. A known weight of each organism, which had previously been blotted with filter paper, was air-dried, desiccated over CaCl_2 , and finally oven-dried; and it was reweighed at each stage. They found that, throughout the whole process, the spores of *Bacillus megaterium* lost 58 % by weight of water and those of *B. cereus* var. *mycoides* 71 %; while the vegetative cells of *B. megaterium* lost 80 %, and those of *B. cereus* var. *mycoides* 88 %. Although the method was almost certainly too crude for these figures to be regarded as being at all reliable absolute values, they do accord with the results of the present investigation in as far as they indicate that the spores contained appreciably less water than the vegetative cells.

Henry & Friedman did not, however, base their final estimation of water content on the overall loss of weight, but only on the percentage loss of weight between the stages of air drying and oven drying. The reason for their doing this is not altogether clear, for although, as they themselves point out, neither the blotted nor the air dried cells were very satisfactory starting materials, the former would seem to have been preferable in so far as the vegetative cells were probably mostly still intact, which they were unlikely to have been after air drying. The conclusion they consequently drew, to the effect that both the spores and the vegetative cells contained similar amounts of water (*c.* 16 %), is almost certainly incorrect.

Powell & Strange (1953) have also expressed the opinion that the water content of spores must be extremely low, and some preliminary weighing experiments of their own, with *Bacillus subtilis*, supported this view.

The Smith interference microscope used in this work was provided out of a grant to one of us (K.F.A.R.) from the London University Central Research Fund.

We are also much indebted to Dr J. R. Baker of the Cytological Laboratory, Department of Zoology, Oxford, and Mr F. H. Smith of Messrs Charles Baker of Holborn for kindly undertaking to read our manuscript and offer useful suggestions.



K. F. A. ROSS AND E. BILLING: THE WATER CONTENT OF BACTERIA AND SPORES.
PLATE 1

(Facing p 425)

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EXPLANATION OF PLATE

Fig. 1 was taken with a Watson phase-contrast microscope with a 2 mm. fluorite objective and a 25 % absorbing 90° positive phase plate. Figs. 2 and 3 were taken with a Smith interference microscope with a 2 mm. 'double focus' objective and an Ilford 807 (mercury green) filter. The scale, indicated at the bottom of the page, is the same for all the photographs.

Fig. 1. Phase-contrast photograph of the spores of *B. cereus* mounted in water. The centres of the spores appear bright, indicating that they are appreciably more refractile than the vegetative cells.

Fig. 2. Photograph with the interference microscope of the spores of *B. cereus* mounted in water with the analyser goniometer set at 145°. At this setting the field appears maximally dark.

Fig. 3. The same preparation as in Fig. 2 with the goniometer analyser set at 80°. A number of the spores now appear maximally dark. The rotation of the analyser from the position in Fig. 2 represents a phase retardation of 130°.

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A Comparison of the Uridine Pyrophosphoglycosyl Metabolism of Capsulated and Non-capsulated Pneumococci

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SUMMARY: A study has been made of the uridine pyrophosphoglycosyl compounds present in a non-capsulated pneumococcus (*Streptococcus pneumoniae*, strain R19, derived from a type II organism) and a capsulated pneumococcus (type III; *Streptococcus pneumoniae*, strain A66), and also of certain enzymes involved in the metabolism of these compounds. It has been found that both the pneumococcal strains contained considerable amounts of uridine pyrophosphoglucuronic acid (UPPGA) and uridine pyrophosphoacetylglucosamine (UPPAG), with lesser amounts of uridine pyrophosphoglucose (UPPG), uridine-5'-monophosphate (UMP), uridine pyrophosphate (UPP) and uridine triphosphate (UTP). The patterns shown by these two strains with respect to uridine nucleotide content were very similar.

Cell-free extracts of strain R19, derived from a type II organism, were obtained; these extracts contained the following enzymes: glucose-6-phosphate dehydrogenase, uridyl transferase, inorganic pyrophosphatase, nucleoside diphosphokinase, hexokinase and phosphoglucomutase. Examination of the strain A66 capsulated (type III) organism showed the presence of uridyl transferase, nucleoside diphosphokinase and inorganic pyrophosphatase.

It is known that the capsule of type III pneumococci is composed of a polysaccharide whose basic repeating unit is cellobiuronic acid, i.e. glucuronido-1- β -4-glucose (Reeves & Goebel, 1941). To date little is known of the mechanism of formation of polysaccharides whose constituent sugars are other than glucose. There is some evidence, however, that the uridine pyrophosphoglycosyl compounds uridine pyrophosphoglucose (UPPG), uridine pyrophosphoacetylglucosamine (UPPAG) and uridine pyrophosphoglucuronic acid (UPPGA) may be involved in the initial stages of polysaccharide formation. Glaser & Brown (1955) demonstrated that UPPGA and UPPAG are involved in the overall synthesis of hyaluronic acid by Rous sarcoma, while Mills & Smith (unpublished observations) have found that the above compounds are participants in the synthesis of the di- and tetrasaccharide units of hyaluronic acid, by using extracts of rat skin or Rous sarcoma. The basic steps of synthesis of certain types of polysaccharides may thus be analogous to the formation of sucrose and trehalose phosphate through the mediation of UPPG (Leloir & Cardini, 1953; Cardini, Leloir & Chiriboga, 1955; Leloir & Cabib, 1953).

While it is well known that pneumococci have an intense glycolytic metabolism (White, 1938) little is known about the enzymic steps involved in the overall process apart from the recent findings of Marmur & Hotchkiss (1955)

which indicated that hexose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase were present in a rough strain (R36A) derived from a type II organism. The present research is designed to study the carbohydrate metabolism of pneumococci, with particular reference to polysaccharide synthesis by type III pneumococci and the participation of uridine pyrophosphoglycosyl compounds in such synthesis. The metabolic pattern of capsulated type III pneumococci has also been compared with that of non-capsulated organisms derived from a type II pneumococcus.

METHODS

The organisms used were: (a) A capsulated *Streptococcus pneumoniae*, type III, strain A66, obtained from the National Collection of Type Cultures. (b) A non-capsulated *S. pneumoniae*, type II, strain R19, kindly supplied by Dr R. D. Hotchkiss, Rockefeller Institute for Medical Research, New York, U.S.A.

Adenosine diphosphate (ADP), triphosphopyridine nucleotide (TPN), 75 % purity, glucose-6-phosphate and hexokinase (type II from yeast) were obtained from Sigma Chemical Company, St Louis, Mo., U.S.A.

Diphosphopyridine nucleotide (DPN), 95 % purity, and glucose-1-phosphate were obtained from C. F. Boehringer and Soehne, Mannheim, West Germany.

Uridine triphosphate (UTP) was obtained from Pabst Laboratories, Milwaukee, Wisconsin, U.S.A.

Bacto Brain Heart Infusion was obtained from Difco Laboratories, Detroit, Michigan, U.S.A.

UPPG and UPPAG were prepared from yeast by the method of Caputto, Leloir, Cardini & Paladini (1950) and separated by ion exchange and paper chromatography.

UPPGA was isolated from guinea-pig liver (Smith & Mills, 1954*a*).

³²P labelled inorganic pyrophosphate was prepared from Na₂H³²PO₄ (from the Radiochemical Centre, Amersham, England) by the method of Kornberg & Pricer (1951*a*).

Phosphoglucomutase was prepared and purified from rabbit muscle by the method of Najjar (1948).

Cultivation and harvesting of organisms

Stock cultures of *Streptococcus pneumoniae*, type III, strain A66, were maintained on meat-extract + 10 % (v/v) oxalated horse-blood agar plates; before use the organism was passaged one to three times through mice to ensure good capsule formation, followed by isolation in pure culture on the blood agar medium. The well-capsulated organisms were washed from this plate with sterile 1 % (w/v) saline and inoculated into requisite volumes of brain heart infusion medium (3.7 %, w/v; pH 7.4) allowing 18 hr. growth at 37° before harvesting; that good capsule formation had occurred was checked by the standard microscopic methods. For enzyme studies, the harvesting of organisms was accomplished by centrifuging at 1500 g for 15 min. and then

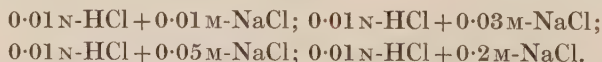
re-centrifuging the loosely packed organisms at 10,000 *g* for 15 min. to obtain a tightly packed mass. For nucleotide analysis the organism was grown in 4 l. batches of brain heart infusion medium, incubated at 37° for 18 hr. and formaldehyde solution (40 %, w/v) to a final concentration of 1 % (w/v) was then added; the culture was centrifuged in a Sharples Super Centrifuge 15 min. after the addition of formaldehyde and the pneumococcal paste suspended in the minimum volume of distilled water. Boiling water extracts for nucleotide analysis were made by pouring the suspension of organisms obtained from each 4 l. batch into 100 ml. vigorously boiling water; boiling was continued for 1 min. and the suspension then rapidly cooled at -15° until a temperature of 0° was attained. Bacterial debris was removed by filtration. Stock cultures of the non-capsulated R19 strain of *Streptococcus pneumoniae* were also maintained on meat-extract + horse-blood (10 %, v/v) agar plates. Before mass cultivation of organisms for nucleotide analysis, two daily subcultures were made in brain heart infusion medium (3.7 %, w/v; pH 7.4). Harvesting for nucleotide analysis and enzyme studies was accomplished by a single centrifugation at 1500 *g* for 20 min. Boiling water extracts were made as described for the A66 strain.

Paper chromatography of the nucleotides was carried out as previously described by Smith & Mills (1954*a*). Paper chromatography of sugars was carried out with the butanol/acetic acid/water solvent of Partridge (1948) and the ethanol/ammonium acetate solvent of Paladini & Leloir (1952) as described for nucleotide chromatography. The sugar spots were developed by the silver nitrate method of Trevelyan, Procter & Harrison (1950).

RESULTS

Uridine nucleotide analysis

Nucleotide analysis of the boiling water extracts of the R19 and A66 strains was confined to the uridine derivatives. The filtered aqueous extracts were adjusted to pH 9 with ammonia and applied to Dowex 1 Cl' columns (10 cm. × 1 cm. diameter for extracts obtained from a 4 l. culture). The columns were washed with 50 ml. distilled water and developed with solutions containing increasing concentrations of HCl and NaCl, namely:



Each of the eluted fractions was collected in bulk and treated by the charcoal adsorption and elution method as described by Smith & Mills (1954*b*) before chromatography in the ethanol/ammonium acetate solvent. As will be seen from Fig. 1 both the capsulated (A66) and non-capsulated (R19) strains of pneumococci possess the uridine nucleotides UMP, UPPG, UPPGA, UPP and UTP. These nucleotides were eluted from the paper chromatograms and specifically identified by the enzymic and hydrolytic methods described by Smith & Mills (1954*a*).

Both strains of pneumococci also contained material chromatographically identical with UPPAG which, on hydrolysis with 0.01 N-H₂SO₄ for 10 min.

yielded UPP and a sugar corresponding to acetylglucosamine in the solvents of Partridge (1948) and of Paladini & Leloir (1952). The positions of some sugars in this latter solvent, hitherto used only for nucleotides, are shown in Fig. 2.

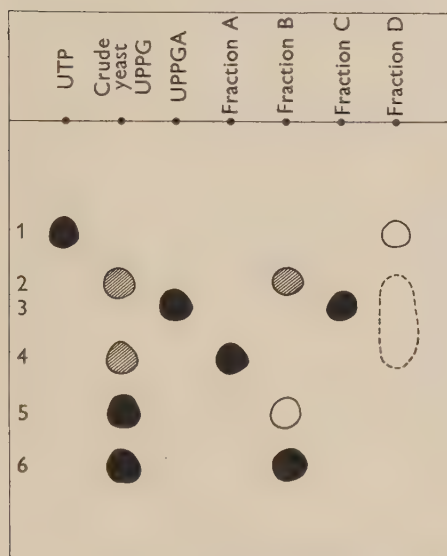


Fig. 1

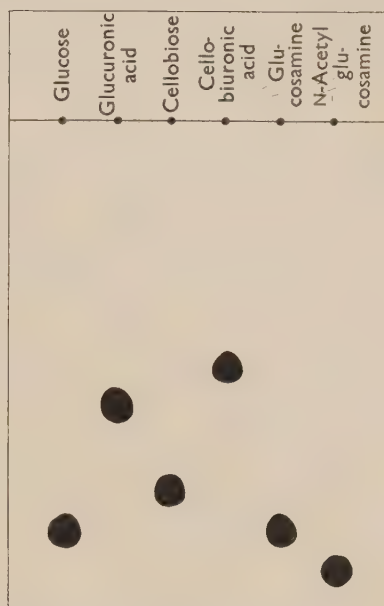


Fig. 2

Fig. 1. Paper chromatography of Dowex 1 Cl⁻ column fractions from aqueous extracts of *Streptococcus pneumoniae*, strain R19 (derived from a type II organism), and *S. pneumoniae*, strain A66 (type III). Whatman no. 1 acid-washed paper and ethanol/ammonium acetate solvent containing 10^{-2} M-versedene were used, and the chromatograms developed for 24 hr. Column eluate fractions were treated as described in text. Fraction A = 0.01 N-HCl + 0.01 M-NaCl eluate; fraction B = 0.01 N-HCl + 0.03 M-NaCl eluate; fraction C = 0.01 N-HCl + 0.05 M-NaCl eluate; fraction D = 0.01 N-HCl + 0.2 M-NaCl eluate. 1, UTP; 2, UPP; 3, UPPGA; 4, UMP; 5, UPPG; 6, UPPAG. A crude yeast UPPG preparation, pure UTP and pure liver UPPGA were used as markers. Intensity of shading of the spots is an indication of the relative amounts of nucleotides present.

Fig. 2. Paper chromatography of some sugars in ethanol/ammonium acetate solvent on Whatman no. 1 paper; development for 18 hr.

The proportional distribution of the uridine nucleotides was comparable in the two strains after 18 hr. of growth; the relative percentage distribution of the uridine nucleotides in the strains may be designated as follows: UPPAG 35 %, UPPGA 30 %, UPPG 7 %, UTP 7 %, UPP 7 % and UMP 14 %. Of the total nucleotides present in the organisms, uridine exceeded adenine in the ratio of 2:1.

The enzymes of the uncapsulated strain R19 of Streptococcus pneumoniae

Several conventional methods for disrupting micro-organisms were tested in an attempt to obtain cell-free preparations for enzyme studies. Lysozyme as used under the conditions specified by Welshimer (1953) for *Bacillus*

megaterium failed to disrupt the organism. While Triton X100 (0.1 %, w/v; Rohm and Hass Co., Philadelphia, Penn., U.S.A.) and bile salts (1 %, w/v, final concentration) dissolved the organism, most of the enzymes under study were irreversibly inactivated by these processes. Freezing and thawing in an ice + salt mixture was also ineffective; the use of ethanol + solid CO₂ for the freezing and thawing technique was successful in breaking down the pneumococci but several enzymes under study were inactivated in the process. The most consistently effective method of disruption was to grind the organisms by hand mortar and pestle in the presence of ballotini (no. 12) and buffers of specific pH values. The use of ballotini in the Mickle disintegrator resulted in considerable loss of enzyme activity.

The centrifuged organisms of the R19 strain (from 50 ml. culture medium) were suspended in three times their weight of M/15 phosphate buffer (pH 6.6), ground in a glass mortar for 20 min. with ten times their weight of ballotini, allowed to stand at 0° for 1–2 hr. and re-ground for 10 min. The extract was centrifuged at room temperature until clear (10,000 g for 5 min.) and the following enzymes were shown to be present.

Glucose-6-phosphate dehydrogenase. The method of Kornberg & Pricer (1951*b*) was used for the assay of this enzyme, with the results shown in Fig. 3.

Uridyl transferase. The presence of an active uridyl transferase was shown by the following reactions:

- (1) $\text{UTP} + \alpha\text{-glucose-1-phosphate} \rightarrow \text{UPPG} + \text{pyrophosphate};$
- (2) $\text{UPPG} + \text{pyrophosphate} \rightarrow \text{UTP} + \alpha\text{-glucose-1-phosphate}.$

The products of reaction 1 were identified chromatographically by methods previously described (Munch-Petersen, Kalckar, Cutolo & Smith, 1953; Smith & Mills, 1954*a*) and the result is shown in Fig. 4. In the normal course of reaction 1 it is essential to add purified extraneous inorganic pyrophosphatase to remove the pyrophosphate formed; it was found however that the pneumococcal extract contained a very potent inorganic pyrophosphatase, allowing the reaction to proceed to the extent shown in Fig. 4.

Reaction 2 was assayed enzymically (Munch-Petersen, Kalckar & Smith, 1955) with the results shown in Fig. 5. Further confirmation of the existence of this reaction was obtained chromatographically by using ³²P-labelled inorganic pyrophosphate as shown in Fig. 6.

In the assay described in Fig. 5, the reaction was triggered with pyrophosphate; it was necessary however to add UPPG to the bacterial extract before adding phosphoglucomutase; when the phosphoglucomutase was added before the UPPG, the overall rate of reaction was decreased by *c.* 60 %. This effect may be due to a competitive inhibition of the uridyl transferase system by a factor present in the phosphoglucomutase preparation.

Pure UPPAG was also pyrophosphorolysed by the enzyme extract to yield ³²P-labelled UTP; this result is shown in Fig. 6. It was also possible to demonstrate this reaction spectrophotometrically using the method employed by Mills, Ondarza & Smith (1954) for the pyrophosphorolysis of UPPAG by liver

nuclei extracts. In the present case, $0.1 \mu\text{mole}$ UPPAG gave a ΔE_{340} value of 0.50 in 30 min. in the presence of $200 \mu\text{l.}$ pneumococcal extract.

Inorganic pyrophosphatase. A $25 \mu\text{l.}$ sample of a typical pneumococcal extract completely hydrolysed $1 \mu\text{mole}$ inorganic pyrophosphate to orthophosphate in 15 min. at 25° . In brain heart infusion medium the R19 pneumococcus strain was capable of growing in the presence of added inorganic pyrophosphate at concentrations of the latter up to 0.002 M ; above this value growth ceased.

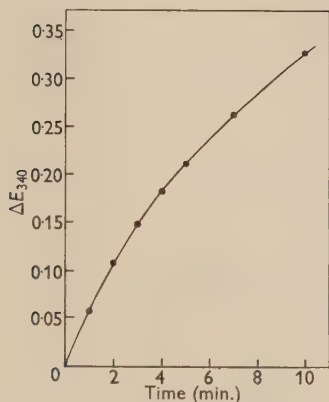


Fig. 3

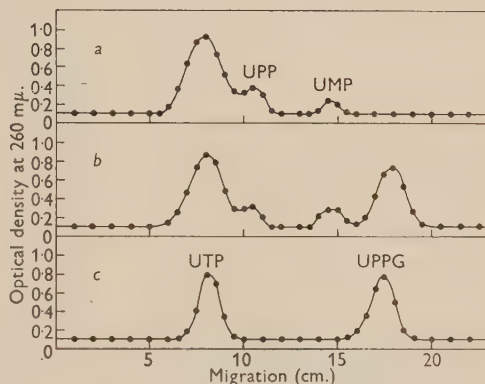


Fig. 4

Fig. 3. The glucose-6-phosphate dehydrogenase activity of an extract of *Streptococcus pneumoniae*, strain R19 (derived from a type II organism). The incubation mixture consisted of: 0.1 M -Tris buffer (pH 7.8); $5 \mu\text{mole}$ MgCl_2 ; $50 \mu\text{l.}$ pneumococcal extract; $0.1 \mu\text{mole}$ glucose-6-phosphate; $0.25 \mu\text{mole}$ TPN; total volume 1.0 ml. Control cuvettes were used simultaneously in which glucose-6-phosphate or pneumococcal extract were omitted; the experimental E_{340} values were corrected for these blank values.

Fig. 4. The formation of UPPG by an extract of *Streptococcus pneumoniae*, strain R19 (derived from a type II organism). (a) $2 \mu\text{mole}$ UTP; $5 \mu\text{mole}$ MgCl_2 , $400 \mu\text{l.}$ pneumococcal extract and 0.1 M -Tris buffer (pH 7.8) to a final volume of 2 ml. ; mixture incubated for 30 min. at 25° . (b) The same as (a) with the addition of $5 \mu\text{mole}$ α -glucose-1-phosphate. Reaction stopped and protein removed with $200 \mu\text{l.}$ 10% (w/v) perchloric acid and centrifugation. Subsequent charcoal adsorption, elution and chromatography of the nucleotides in the ethanol/ammonium acetate solvent was carried out as described by Smith & Mills (1954a). Chromatograms were scanned in the Unicam S.P. 500 spectrophotometer at $260 \text{ m}\mu$. (c) Pure marker substances chromatographed simultaneously.

Nucleoside diphosphokinase. Nucleoside diphosphokinase, catalysing the reaction $\text{UTP} + \text{ADP} \rightleftharpoons \text{UPP} + \text{ATP}$ (described by Berg & Joklik, 1954, for yeast and rabbit muscle) was shown to be present in the pneumococcus R19 extract by chromatographic means; data for this experiment are shown in Fig. 7.

Hexokinase. Extraction of hexokinase was achieved by grinding the organisms with $\text{M}/15$ phosphate buffer (pH 6.6). The assay was conducted spectrophotometrically by the method of Slein, Cori & Cori (1950) and the results are indicated in Fig. 8.

Phosphoglucomutase. While buffered extracts of the R19 strain of pneumococcus did not yield a very active phosphoglucomutase preparation, the

enzyme was nevertheless shown to be present by the conversion of glucose-1-phosphate to glucose-6-phosphate, the production of the latter being determined by the spectrophotometric method of Kornberg & Pricer (1951*b*). Figure 9 records the data for this reaction.

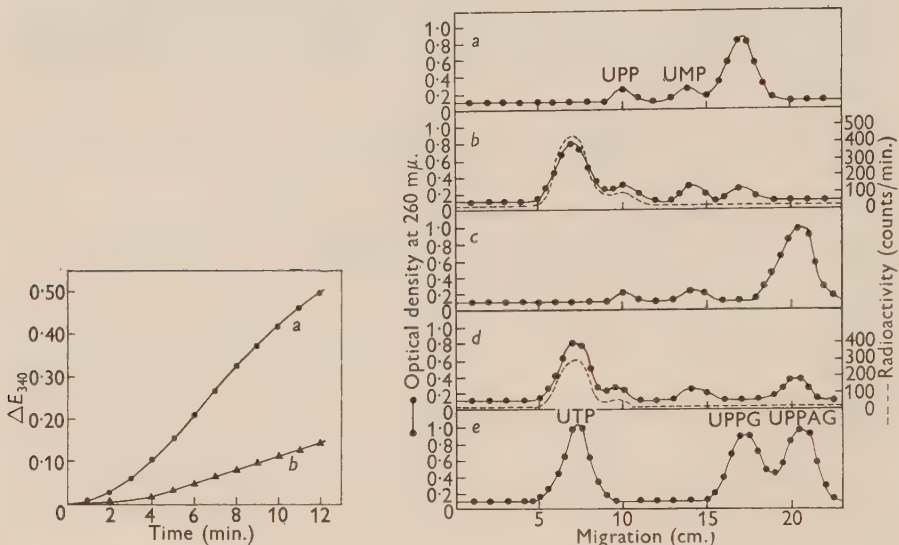


Fig. 5

Fig. 6

Fig. 5. The pyrophosphorolysis of UPPG by an extract of *Streptococcus pneumoniae*, strain R 19 (derived from a type II organism). 0.3 μ mole UPPG was incubated with 100 μ l. pneumococcal extract, and 5 μ mole MgCl_2 , 0.25 μ mole TPN, 2.5 μ mole cysteine, 30 μ l. phosphoglucomutase preparation, 1.0 μ mole potassium pyrophosphate, 0.1 M-Tris buffer (pH 7.8); final volume 1 ml. Curve *a*, UPPG added to enzyme mixture before phosphoglucomutase. Curve *b*, UPPG added to enzyme mixture after phosphoglucomutase. Reaction started with pyrophosphate in both cases. Control cuvettes were run simultaneously, in which UPPG or pneumococcal extract were omitted; experimental E_{340} values were corrected for these blank values.

Fig. 6. The pyrophosphorolysis of UPPG and UPPAG by extracts of *Streptococcus pneumoniae*, strain R 19 (derived from a type II organism). (a) 2 μ mole UPPG incubated with 5 μ mole MgCl_2 , 400 μ l. pneumococcal extract and 0.1 M-Tris buffer to a final volume of 2 ml., for 30 min. at 25°. (b) Same as (a) with the addition of 5 μ mole potassium pyrophosphate labelled with ^{32}P (1 μ c.). (c) Same as (a) with 2 μ mole UPPAG replacing the UPPG. (d) Same as (b) with 2 μ mole UPPAG replacing the UPPG. Reaction mixtures treated as Fig. 4. In reactions (b) and (d) the charcoal used was previously washed with inorganic phosphate and pyrophosphate. (e) Pure marker substances chromatographed simultaneously. In addition to scanning at 260 m μ . in the Unicam S.P. 500 spectrophotometer, the chromatograms were scanned for radioactivity as previously described (Smith & Mills, 1954*a*).

The most effective pH value for the extraction of the above mixture of enzymes from the non-capsulated R 19 pneumococci was pH 6.6. Small differences in acidity or alkalinity effectively decreased the extractive properties of the buffer. When, however, R 19 pneumococci were ground with distilled water in the manner described, glucose-6-phosphate dehydrogenase could be selectively extracted. This is particularly useful in view of the desirability of

having a glucose-6-phosphate dehydrogenase preparation free from uridyl transferase, since the majority of preparations of the former usually have an associated uridyl transferase. Such a bacterial extract should thus be of considerable use in determining the presence or absence of uridyl transferase in various tissues.

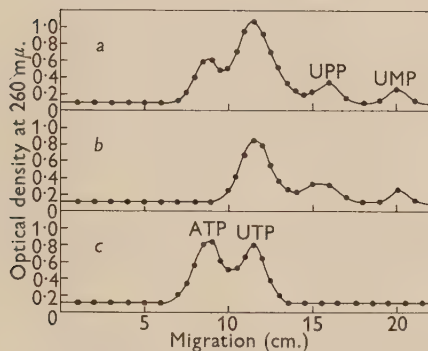


Fig. 7

Fig. 7. The formation of ATP from UTP and ADP by an extract of *Streptococcus pneumoniae*, strain R 19 (derived from a type II organism). (a) 1 μ mole UTP incubated with 5 μ mole $MgCl_2$, 150 μ l. pneumococcal extract, 1 μ mole ADP and 0.1 M-Tris buffer (pH 7.8) to a final volume of 1 ml., for 30 min. at 25°. (b) Same as (a) without the addition of ADP. Reaction mixture treated as in Fig. 4. (c) Pure marker substances chromatographed simultaneously.

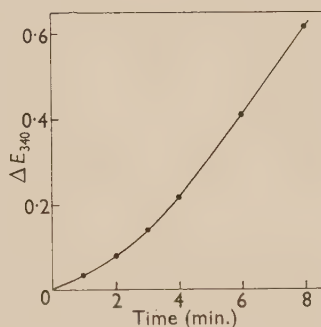


Fig. 8

Fig. 8. The hexokinase activity of an extract of *Streptococcus pneumoniae*, strain R 19 (derived from a type II organism). The incubation mixture consisted of: 100 μ l. pneumococcal extract; 5 μ mole $MgCl_2$; 0.25 μ mole TPN; 1 μ mole glucose; 0.2 μ mole ATP; 0.1 M-Tris buffer pH 7.8 to a final volume of 1 ml. Control cuvettes were run simultaneously in which ATP or pneumococcal extract were omitted; the experimental E_{340} values were corrected for these blank values.

From the results recorded in Fig. 4 and 6 it is apparent that there was very little breakdown of UTP to UPP or UMP. When, however, 0.1 M-maleate buffer (pH 6.6) was substituted for phosphate buffer in the extraction procedure, UTP was readily broken down to diphosphate and monophosphate under the conditions specified in Fig. 4.

Enzymes of Streptococcus pneumoniae, type III, strain A 66

The problem of obtaining an active enzyme extract from *Streptococcus pneumoniae*, strain A 66, is made more difficult by the capsules which surround the organisms. So far it has proved impossible to disrupt these pneumococci by any of the methods tested with the uncapsulated strain R 19; in consequence all enzyme studies have been made with intact organisms. The wet packed organisms (10,000 g, 15 min.) were suspended in an equal volume of appropriate buffer and this suspension used in the enzyme experiments. The following enzymes associated with uridine pyrophosphoglycosyl metabolism were shown to be present.

Uridyl transferase. Using ^{32}P -labelled pyrophosphate, pyrophosphorolysis of UPPG to UTP was effectively accomplished by whole cells. The end products of the reaction were identified chromatographically; full data for the experiment are shown in Fig. 10. The UTP formed in the reaction was eluted from paper and further identified by the enzymic assay of Berg & Joklik (1954).

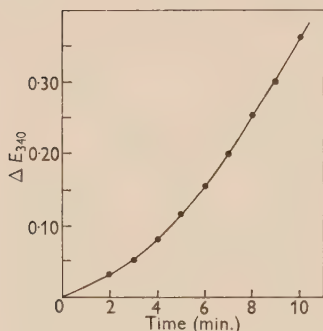


Fig. 9

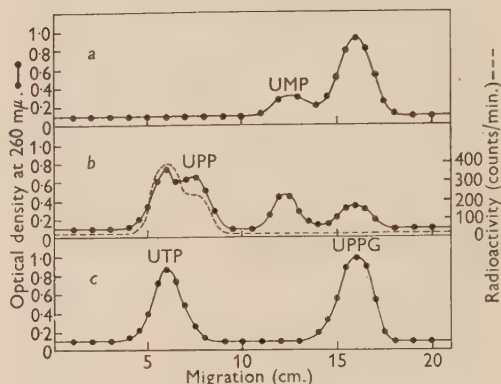


Fig. 10

Fig. 9. The phosphoglucomutase activity of an extract of *Streptococcus pneumoniae*, strain R19 (derived from a type II organism). The incubation mixture contained: 200 μl . pneumococcal extract; 0.1 μmole glucose-1-phosphate; 5 μmole MgCl_2 ; 2.5 μmole cysteine; 0.25 μmole TPN; 0.1 M-Tris buffer pH 7.8 to a final volume of 1 ml. Control cuvettes were run simultaneously in which glucose-1-phosphate or pneumococcal extract were omitted; the E_{340} values were corrected for these blank values.

Fig. 10. The pyrophosphorolysis of UPPG by a suspension of *Streptococcus pneumoniae*, type III, strain A66. (a) 3 μmole UPPG incubated with 10 μmole MgCl_2 , 0.5 ml. pneumococcal suspension in 0.1 M-Tris buffer (pH 7.8), and 0.1 M-Tris buffer pH 7.8 to a final volume of 1.5 ml. (b) Same as (a) with the addition of 5 μmole potassium pyrophosphate labelled with ^{32}P (1 μc .). Reaction mixtures treated as in Fig. 6. (c) Pure marker substances chromatographed simultaneously.

Nucleoside diphosphokinase. This enzyme was shown to be present in the whole cells by chromatographic means; the method employed was identical with that described in Fig. 7 with, however, the substitution of 0.5 ml. pneumococcal type III suspension for the extract used in the case of the uncapsulated R19 strain. The results obtained were essentially similar to those shown in Fig. 7.

Inorganic pyrophosphatase. As with strain R19, the formation of UPPG from UTP and α -glucose-1-phosphate by the capsulated A66 pneumococci proceeded without the addition of extraneous inorganic pyrophosphatase; an active inorganic pyrophosphatase was found in the intact organisms of strain A66. When grown in brain heart infusion medium it was apparent that the capsulated and non-capsulated pneumococci were identical with respect to pyrophosphate tolerance, the capsulated A66 strain growing at all concentrations of pyrophosphate up to 0.002 M; above this figure growth again ceased entirely.

DISCUSSION

While comparatively little is known of the mechanism of synthesis of polysaccharides other than those containing only glucose, it is not improbable (see introduction) that such synthesis may be mediated through the uridine pyrophosphoglycosyl compounds. The uridine pyrophosphoglycosyl metabolism of the pneumococci used here may well bear some relation to the synthesis of the constituent polysaccharides. The only known mechanism for the intramolecular transfer of glucuronic acid is that mediated by UPPGA (Smith & Mills, 1954*a*; Mills & Smith, 1955), and it is conceivable, in view of the presence of this compound in the pneumococci, that UPPGA may act as a transferring agent for glucuronic acid. In the metabolism of type III pneumococci the glucuronic acid acceptor may well be glucose, giving rise to the repeating disaccharide unit of the capsular polysaccharide.

In view of the fact that both the capsulated and non-capsulated strains contain virtually identical enzyme systems of uridine nucleotide metabolism and also exhibit the same pattern of uridine nucleotide distribution, it is possible that the difference in their ability to produce the capsular polysaccharide resides in a subsequent conjugation step or an even later polymerization step.

It has been shown above that both the strains of pneumococcus used contain considerable amounts of UPPAG. Since the type III capsular polysaccharide is completely devoid of acetylglucosamine, the uridine derivative of this sugar may be involved in the synthesis of the cellular polysaccharide as distinct from the capsular polysaccharide. Preliminary studies by the present authors on the sugar composition of the cellular polysaccharide of strain R19 derived from a type II organism, have indicated that this material is rich in glucosamine which is possibly the *N*-acetyl derivative.

It was shown by Strominger, Kalckar, Axelrod & Maxwell (1954) that the mechanism of formation of UPPGA by liver tissue occurs by means of a DPN-linked oxidation of UPPG. The presence of an active system for the synthesis of UPPG in both strains of pneumococci suggests that UPPGA may well be derived from UPPG in these micro-organisms. It has not yet proved possible to demonstrate conclusively the presence of a UPPG oxidase which causes the formation of UPPGA in these bacteria; this, however, does not preclude the possibility that such an enzyme system exists.

While it has been possible to obtain cell-free enzyme systems from the non-capsulated R19 strain (derived from type II) by fairly simple methods, the capsulated A66 strain (type III) has remained resistant to the techniques employed; the difficulty in disrupting this strain is probably due to the presence of the capsule. A method which may yield the desired results would be a removal of the capsule by a specific enzyme technique; Sickles & Shaw (1934) and Dubos (1935) obtained enzyme preparations from *Bacillus palustris* and a similar organism, which were capable of hydrolysing specifically the type III pneumococcal capsular polysaccharide; this would prove a useful tool in rendering the organism more liable to disruption. The principles set out above will be the basis of further investigations.

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Neurospora crassa Mutants Lacking Argininosuccinase

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SUMMARY: Four mutant strains of *Neurospora crassa*, due to repeated mutation within the same short chromosome region, required arginine as a nutrient and failed to respond to citrulline. These strains accumulated argininosuccinic acid in the mycelium when grown on a medium supplemented with arginine. When the medium also contained citrulline this accumulation of argininosuccinic acid was enhanced, and citrulline also accumulated in the mycelium. Citrulline depressed the growth of the mutants at a concentration at which it had no effect on growth of the wild type.

Cell-free extracts of the wild type contained an argininosuccinase which catalysed the reversible splitting of argininosuccinic acid to arginine and fumaric acid. A procedure is described for the approximately fivefold purification of the enzyme. The pH optimum for the synthesis of argininosuccinic acid from arginine and fumaric acid was close to pH 7.0.

Neither cell-free extracts nor partially purified preparations from the four mutants showed any trace of argininosuccinase activity; 1–2 %, and 0.2 %, of normal wild-type activity should have been detectable in crude cell-free extracts and purified preparations, respectively. Neither the argininosuccinase activity of crude wild-type extracts, nor that of the partially purified preparations was appreciably altered by the addition of corresponding preparations from one of the mutants. A mixture of wild-type and mutant mycelium extracted together yielded the amount of activity to be expected on the assumption that the mutant mycelium was neither contributing to, nor decreasing, the yield.

Arginase and fumarase activities were similar in the wild type and one of the mutants.

The great majority of nutritionally exacting mutants of *Neurospora crassa* and other micro-organisms are enabled to grow by the addition to the minimal medium of a single substance. This has suggested that 'a large class of genes exist in which each gene controls the synthesis of, or the activity of, but a single enzyme' (Horowitz, 1950). Mutants which require arginine or one of its precursors (Srb & Horowitz, 1944) have long provided one of the most-quoted examples of a series of mutational 'blocks' in a biosynthetic sequence, but a more direct verification of the interpretation through an investigation of the enzymes involved has not, until recently, been possible. Recent work by Ratner and her collaborators (Ratner & Petrack, 1953*a, b*; Ratner, Petrack & Rochovansky, 1953; Ratner, Anslow & Petrack, 1953) has elucidated the mechanism of the conversion of citrulline to arginine in liver and kidney, and suggested the possibility of parallel investigations on some of the arginine-requiring *Neurospora* mutants. The present study is concerned with a class of mutants located in linkage group VII (Newmeyer, 1957). Preliminary experiments by Dr Dorothy Newmeyer (personal communication) suggested that these mutants were 'blocked' in the terminal step in arginine synthesis, the splitting of argininosuccinic acid to give arginine and fumaric acid. The enzyme catalysing this reaction, which was called the splitting enzyme by Ratner, will be referred to in the present paper as argininosuccinase.

METHODS

Strains. The wild-type strain STA of *Neurospora crassa*, and the arginine-requiring mutant strains B317, B362, B368 and B370 were supplied by Dr Dorothy Newmeyer, and were the same as those used in her parallel genetic investigation (Newmeyer, 1957). In a few experiments the genetically and biochemically distinct arginine-requiring mutant 36703, re-isolated in this laboratory from an ascospore, was used.

Culture methods. The minimal medium used throughout was Fries no. 3 (composition given in Fincham, 1954). For growth of the mutants (and also of the wild type where comparisons between wild type and mutant were being made) it was supplemented with L-arginine monohydrochloride to 2.5×10^{-3} M. Cultures were grown from conidial inocula without agitation at 25°. Mycelium for enzyme extraction was grown in culture flasks each containing 150 ml. of medium at a depth of about 1 cm., while in other experiments 15 or 40 ml. media in 100 and 250 ml. flasks, respectively, were used.

Identification of amino acids in mycelial extracts. Mycelial pads were washed with water, sucked as dry as possible on a Buchner funnel, and extracted with their own weight of 0.03 M-phosphate buffer (pH 7.5) for 10 min. at 100°. Samples (2 μ l.) of the resulting extracts were applied to Whatman no. 1 paper sheets and the chromatograms developed for 8 hr. with phenol saturated with water in the presence of ammonia vapour. The papers were dried at room temperature and coloured with ninhydrin in the usual way. Provisional conclusions drawn from the phenol chromatograms were checked by two-dimensional chromatography using butanol+acetic acid (100 n-butanol:24 glacial acetic acid:100 water, by vol.; upper phase) as the second solvent. Identifications of the major amino acid spots were made by two-dimensional chromatography of samples of the unknowns with and without the addition of appropriate amounts of known amino acids.

Preparation and estimation of argininosuccinic acid. Barium argininosuccinate was prepared from arginine and fumarate with partially purified pig kidney enzyme as described by Ratner, Petrack & Rochovansky (1953). The barium salt was converted to the potassium salt, which was standardized by reference to leucine by the quantitative ninhydrin procedure of Moore & Stein (1948). Assuming two equivalents of barium, the product was 65% pure and, from its absorption at 240 $m\mu$., appeared to contain a molecular proportion of 10% of fumaric acid. No amino acid impurities were revealed on phenol water chromatograms; the argininosuccinic acid gave a compact spot at R_F 0.27. Argininosuccinic acid formed enzymically in experimental reaction mixtures was separated from arginine and ornithine (the only other amino acids present in appreciable concentration) on phenol chromatograms. It was determined quantitatively by scanning the argininosuccinic acid region of each sheet parallel to the solvent front with an EEL scanner (made as part of the EEL electrophoresis apparatus; Evans Electroselenium Ltd., Harlow, Essex) and comparing the heights of the peaks on the scanning diagram with a standard curve obtained from a series of argininosuccinate standards run on the

same sheets as the unknowns. The method was essentially the same as described elsewhere (Fincham & Boulter, 1956) for glutamic acid except that scanner readings were corrected individually by subtracting the average reading given by the blank paper on each side of each spot. The error of the method was not greater than 5% for concentrations of argininosuccinate of 6×10^{-3} M or more, and a concentration of 10^{-4} M could be easily detected by superimposing samples three times on the same spot.

Other analytical procedures. Citrulline was determined by the method of Archibald (1944) with the omission of the procedures designed to get rid of allantoin and urea. It was found that incubation of mycelial extracts or used medium with urease made no difference to the results; extracts of mycelium which had not been grown in the presence of citrulline contained no substances reacting to give a colour with diacetyl monoxime.

Protein was determined by the quantitative biuret method previously described (Fincham, 1954).

Ammonia was determined by microdistillation in Conway units, followed by nesslerization.

pH values were measured with a glass electrode.

Enzyme assays. Argininosuccinase was determined by measuring the argininosuccinic acid produced from arginine and fumaric acid in the following system: 0.05 M-sodium fumarate; 0.05 M-L-arginine monohydrochloride; 0.025 M-phosphate ($\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$) at pH 7.5; 0.2 ml. enzyme solution in a total vol. of 0.8 ml.; temp. 35°. The reaction was stopped by brief heating to 100°, duplicate (or, in critical experiments, quadruplicate) samples were applied to chromatograms, and argininosuccinic acid determined by the method described above. In experiments designed to detect traces of enzyme activity, control tubes lacking fumarate were set up, and the small amount of apparent argininosuccinic acid in the control (due to a very faint background streak apparently deriving from the enzyme preparation) was subtracted from the corresponding value for the complete system in each case. A possible weakness of the method, especially when used to assay crude extracts, is that arginine and fumarate concentrations will tend to be decreased by arginase and fumarase. It was found, however, that either arginine or fumarate could be decreased in concentration by at least 60% without appreciably affecting the rate of argininosuccinic acid formation during the incubation periods usually used. The fact that argininosuccinic acid formation was nearly proportional to enzyme concentration even in the case of crude extracts (Table 3) indicated that there was probably always a sufficient excess of substrate.

Fumarase was determined by following the disappearance of fumaric acid in a Unicam ultraviolet spectrophotometer at 240 m μ . (Racker, 1950).

Arginase was determined qualitatively by observing the appearance of ornithine on chromatograms, or quantitatively by determination of ammonia after treatment of digests with excess urease (Fig. 7). The urease was obtained from L. Light and Co. (Colnbrook, Bucks.), and was free from arginase.

Enzyme preparations. For the preparation of cell-free extracts, mycelium from 48 hr. cultures was homogenized with 5–10 times its wet weight of

0.02 M-phosphate ($\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$; pH 7.5) either in an 'Atomix' blender or, in most experiments, in a mortar with powdered glass, and the blend was filtered through kieselguhr to remove cell-wall fragments and particles, as previously described (Fincham, 1954). The filtrate was usually dialysed for 2–3 hr. with stirring, against two changes of the same buffer as used in the extraction.

The following procedure, based partly on unpublished information from Dr Newmeyer and partly on Ratner's method for the pig kidney enzyme, was used to obtain an approximately five-fold purification (with respect to protein) of argininosuccinase from the crude extract. Solid ammonium sulphate (7 g.) was added to 20 ml. of crude extract to give 50 % saturation. This and subsequent ammonium sulphate additions were made over a 15–20 min. period with stirring, the temperature being kept near 0°. The resulting precipitate was collected by centrifugation at 3000 g for 10 min. and taken up in 20 ml. of water. The solution was brought to c. pH 5.5 by the addition of 1.7 ml. 0.1 M- KH_2PO_4 , heated to 51° by immersion, with constant stirring, in a 60° water bath (this took about 3 min.) and cooled in an ice bath. Centrifugation at this stage seemed to be of no advantage. The pH value was brought back to c. 7.5 by the addition of 8.3 ml. 0.1 M- Na_2HPO_4 and solid ammonium sulphate was added as before to 30 % saturation. The fairly bulky precipitate, containing much protein denatured during the heat step, was removed by centrifugation and the supernatant fluid was brought to 55 % saturation with ammonium sulphate. The precipitate was centrifuged down, taken up in 1 ml. of 0.02 M-phosphate buffer (pH 7.5), dialysed with stirring for 2–3 hr. against two changes of the same buffer, and recentrifuged. The slight precipitate was discarded. The supernatant fluid contained 10–15 % of the original protein of the crude extract and about 50–60 % of the original argininosuccinase activity. Most of the arginase and fumarase activities were removed by the first precipitation and the heat step. The final preparation contained not more than 2 % of the original degree of fumarase activity and a variable amount of arginase activity ranging from nearly zero up to about 20 % of that originally present. This preparation was often kept frozen overnight without apparent loss of activity.

RESULTS

Growth responses to arginine and to argininosuccinic acid

The mutants B317, B362, B368 and B370 were practically identical in their responses to arginine (Fig. 1). At lower arginine concentrations the amount of growth made was proportional to the amount of arginine present. An optimum concentration of arginine was 2.5×10^{-3} M, and this was adopted for routine growth of the mutants. At this concentration of arginine growth of the mutants was closely similar to that of the wild type, both in weight of mycelium (Table 1) and in the final pH value of the medium (4.65–4.70 after 2 days for both the mutants and wild type in one experiment). Neither strain B362 nor strain 36703 (which produces normal amounts of argininosuccinase) showed more than an unweighable trace of growth on minimal medium supplemented with

potassium argininosuccinate at concentrations ranging from 10^{-3} M to 5×10^{-3} M.

Table 1. *Inhibition of mutant strains of Neurospora crassa by citrulline*

Growth for 64 hr. at 25° in 15 ml. media. Dry weights to the nearest mg. Concentrations of amino acids 2.5×10^{-3} M in each case.

Medium	Expt. 1		Expt. 2				
	Wild		Wild				
	type	B362	type	B317	B362	B368	B370
	Amount of growth (mg. dry wt.)						
Minimal	58	0	57	0	0	0	0
Minimal + arginine	57	53	64	61	52	55	54
Minimal + arginine + citrulline	63	39	66	45	45	46	35

Accumulation of argininosuccinic acid by mutant mycelium

Two-dimensional chromatograms of extracts of wild-type mycelium grown on minimal medium showed a rather consistent pattern of amino acid spots which was not obviously altered by supplementation of the medium with either L-arginine or arginine + L-citrulline (each amino acid at 2.5×10^{-3} M). The main amino acids present were alanine, glutamic acid and glutamine; several other amino acids were generally present in much lower concentration.

Extracts of the mutant strains B317, B362, B368 and B370 grown on arginine-supplemented medium contained, in addition to the usual amino acids of the wild-type extracts, another major constituent which was identified as argininosuccinic acid. Mycelium of all four mutant strains grown in medium supplemented with both arginine and citrulline showed a strikingly different amino acid pattern. The main acids present were citrulline and argininosuccinic acid, the latter in considerably higher concentration than in mutant mycelium grown without the citrulline supplement. Alanine, glutamic acid and glutamine were all very much decreased in concentration in comparison with extracts of wild-type mycelium, and were often barely detectable. The results of a typical experiment in which one of the mutant strains was compared with wild type are shown in Fig. 2.

It seemed of some interest to determine whether all the citrulline added to mutant cultures could be accounted for at the end of growth either as residual citrulline or as argininosuccinic acid. In one experiment 100 μ mole of L-citrulline was added to 40 ml. of medium which was also supplemented with arginine. After 65 hr. of growth B362 mycelium (*c.* 77 mg. dry weight) was found to contain 18 μ mole of citrulline and 15–20 μ mole (determined by visual comparison with standards on chromatograms) of argininosuccinic acid. The medium contained 56 μ mole of citrulline and no detectable argininosuccinic acid (although up to *c.* 10 μ mole might have been present without being detected on the chromatograms). These figures indicate an accumulation and retention of both amino acids by the mycelium against considerable concentration gradients. A parallel control culture without the citrulline supplement accumulated rather less than 5 μ mole of argininosuccinic acid in the mycelium

and no detectable citrulline either in the medium or the mycelium. Thus although some relatively slight utilization or degradation of the citrulline supplied was not excluded, at least some 85–90% of the citrulline added could be accounted for, either as citrulline or as argininosuccinic acid.

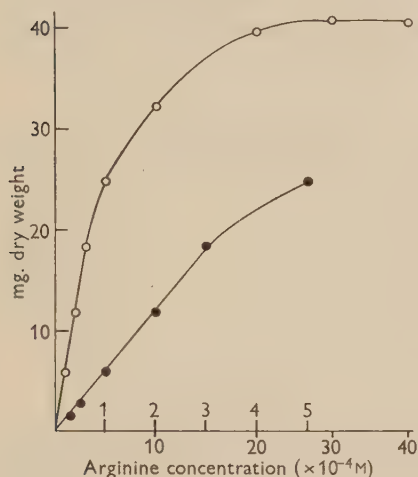


Fig. 1

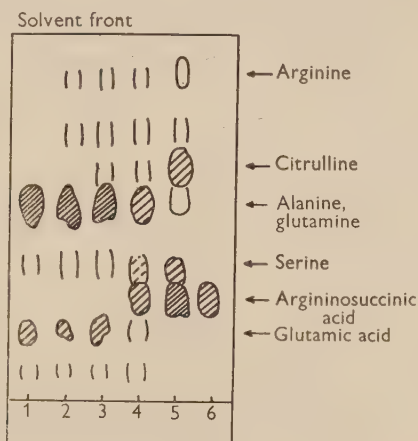


Fig. 2

Fig. 1. Response of *Neurospora crassa* mutant strain B362 to arginine. Growth for 63 hr. at 25° in 15 ml. media. The weights shown are averages of duplicates. Open circles refer to the lower arginine concentration scale, and solid circles to the upper scale. Mutants B317, B368 and B370 gave the same curve to within experimental error.

Fig. 2. Phenol water chromatograms of mycelial extracts from (1) wild type grown on minimal medium; (2) wild type on minimal+arginine; (3) wild type on minimal+arginine+citrulline; (4) mutant B362 on minimal+arginine; (5) mutant B362 on minimal+arginine+citrulline; (6) 5×10^{-3} M argininosuccinate. Relative densities of the major spots are roughly indicated by cross-hatching; faint and streaky spots are not cross-hatched. The arrows indicate the positions of markers. The spots in the alanine region are due to roughly equal amounts of alanine and glutamine; the spot in the serine position in (5) is due partly to an unknown amino acid having a much lower R_F value in butanol+acetic acid than has serine. Mutants B317, B368 and B370 gave essentially similar results.

Characteristics of argininosuccinase from wild type mycelium

Dialysed cell-free extracts of wild-type mycelium could always be shown to contain an argininosuccinase. The presence of arginine in the medium was not found to have any effect on the production of the enzyme. The fractionation procedure already described resulted in an increase in argininosuccinase activity per unit wt. of protein of about five-fold, and, at the same time, generally decreased the arginase and fumarase activities to low values. The effectiveness of the procedure did not appear to be affected by the presence of arginine in the medium used for growing the mycelium. The pH optimum for synthesis of argininosuccinic acid was near 7.0 (Fig. 3). An attempt was made to determine the approximate Michaelis constants for arginine and fumaric acid, but these appeared to be too low to be conveniently determined by the present methods. When either arginine or fumaric acid was decreased in

concentration to 0.01 M argininosuccinic acid formation still proceeded at well over half the maximal rate. Thus the *Neurospora* enzyme appeared to have a higher affinity for both substrates than did the liver enzyme studied by Ratner, Anslow & Petrack (1953). With a partially-purified preparation in which fumarase activity was low it was possible to follow fumaric acid formation from argininosuccinic acid by making use of its absorption in the ultra-violet (Ratner & Petrack, 1953*b*). As would be expected from the value of the

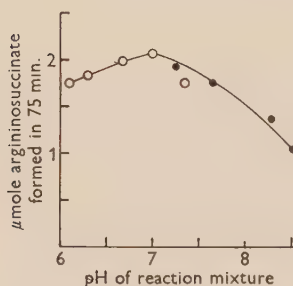


Fig. 3

Fig. 3. Dependence of argininosuccinic acid formation on pH value. Reaction mixtures contained, in 0.4 ml.: 20 μmole sodium fumarate; 10 μmole phosphate (○) or borate (●); 20 μmole L-arginine monohydrochloride; 0.2 mg. protein of partially purified argininosuccinase from wild type. Temperature 35°.

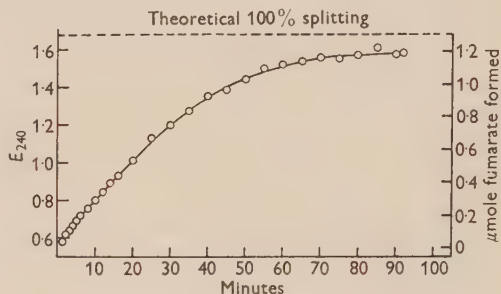


Fig. 4

Fig. 4. Splitting of argininosuccinate by a partially purified enzyme preparation. Spectrophotometer (1 cm.) cell contained, in 2.7 ml.: 50 μmole phosphate (pH 7.5); 1.6 mg. protein; and (added at time 0) 1.3 μmole potassium argininosuccinate. The right-hand scale shows μmole fumarate formed on the assumption that the increase in optical density is due to fumarate formation. Temperature *c.* 18°.

equilibrium constant of the reaction determined by Ratner, Petrack & Rochovansky (1953), the splitting of argininosuccinic acid proceeded almost to completion at the low concentration used in this experiment (Fig. 4). One partially purified enzyme preparation was found to catalyse argininosuccinic acid splitting at the rate of 1.1 μmole/hr./mg. protein at 18° with an argininosuccinate concentration of 0.75×10^{-3} M, and argininosuccinic acid formation at the rate of 4.0 μmole/hr./mg. protein at 35° with arginine and fumarate concentrations of 0.05 M.

Absence of argininosuccinase in the mutants

Table 2 shows the results of one of several experiments in which argininosuccinase activities of wild-type preparations were compared with those of corresponding preparations from mutant mycelium. Neither crude extracts of the mutants, nor mutant preparations which had been fractionated by the same procedure as gave a fivefold increase in wild-type enzyme activity, showed any trace of activity. In the experiment recorded in Table 2, 2% of the activity found in the wild type should have been detectable in the crude extracts of the mutants had it been present, while as little as 0.2% of wild-type activity should have been detectable in the case of the partially purified preparations.

Table 2. *Argininosuccinase assays on crude and partially-purified preparations from mutants and wild type Neurospora crassa*

Reaction mixtures contained, in 0.8 ml.: 40 μ mole L-arginine; 40 μ mole sodium fumarate (replaced by water in controls); 20 μ mole phosphate; and the amounts of protein indicated. Temperature 35°; pH 7.5.

Strain	Type of preparation	Amount of protein (mg.)	Incubation period (min.)	Arginino-succinate formed (μ mole)
Wild type	Crude dialysed extract	1.0	90	0.5
B317	Crude dialysed extract	1.2	180	< 0.02*
B362	Crude dialysed extract	1.3	180	< 0.02*
B368	Crude dialysed extract	1.2	180	< 0.02*
B370	Crude dialysed extract	1.5	180	< 0.02*
Wild type	Partially purified	1.8	45	3.5
B317	Partially purified	2.1	180	< 0.02*
B362	Partially purified	2.2	180	< 0.02*
B368	Partially purified	1.3	180	< 0.02*
B370	Partially purified	2.1	180	< 0.02*

* Chromatogram obtained after three superimpositions of reaction mixture had no visible spot in the argininosuccinic acid region, and gave a scanning diagram the same as for the control without fumarate to within one scanner unit; 0.05×10^{-3} M-argininosuccinate solution treated similarly gave a visible spot and a peak on the scanning diagram 5 units high.

Table 3. *Absence of inhibition of wild-type argininosuccinase by inactive preparations from mutants*

Assay system as for Table 2. Mutant B362 used throughout. The figures show amounts of protein in mg.

Tube no.	Enzyme preparation				Period of incubation (min.)	Amount of arginino-succinic (μ mole)
	Crude dialysed extract		Partially purified preparation			
	Organism					
	Wild type	Mutant	Wild type	Mutant		
1	1.0	—	—	—	120	2.2
2, 2a*	0.5	—	—	—	120	1.2 \pm 0.08†
3, 3a*	0.5	0.6	—	—	120	1.2 \pm 0.03†
4	—	—	1.1	—	40	3.0
5, 5a	—	—	0.55	—	40	1.6 \pm 0.05†
6, 6a	—	—	0.55	0.8	40	1.6 \pm 0.02†

* Duplicate tubes; results averaged.

† Standard error of mean of six determinations.

The possibility that there was a potent inhibitor of argininosuccinase in the mutants appears to be ruled out, at least in the case of mutant B362, by experiments which showed that the addition of either crude or fractionated mutant extracts to corresponding wild-type preparations had no detectable effect on the argininosuccinase activity (Table 3). Furthermore, when mutant mycelium was added to a roughly equal quantity of wild-type mycelium, and the two were put through the whole extraction and fractionation procedure

together, the recovery of argininosuccinase activity was similar to what would be expected if the mutant mycelium were neither contributing to, nor diminishing, the yield of enzyme (Fig. 5).

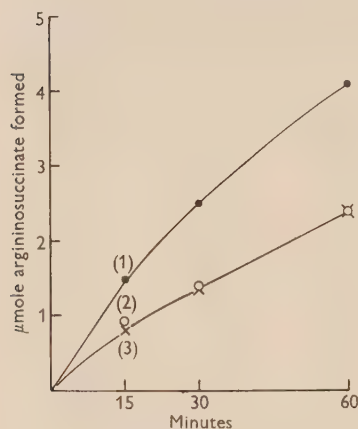


Fig. 5

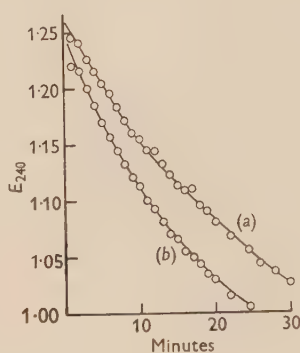


Fig. 6

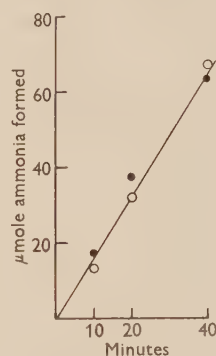


Fig. 7

Fig. 5. Formation of argininosuccinic acid by partially purified enzyme preparations from *Neurospora crassa* wild type (curves 1 and 2) and a mixture of approx. 60% wild type with 40% mutant B362 mycelium (curve 3). Digests contained, in 0.8 ml.: 40 μmole L-arginine hydrochloride; 40 μmole sodium fumarate; 24 μmole phosphate (pH 7.4); and 1.2, 0.6 and 0.9 mg. protein in curves 1–3, respectively. Temperature 35°.

Fig. 6. Fumarase activities of crude extracts of (a) wild type, and (b) mutant B362. Spectrophotometer cells (1 cm.) contained, in 2.7 ml.: 50 μmole phosphate (pH 7.5); 0.5 μmole sodium fumarate (added at time 0); and 0.7 and 0.75 mg. protein in (a) and (b), respectively. Temperature approx. 18°.

Fig. 7. Arginase activities of crude extracts of wild type (○) and mutant B362 (●). Stopped test tubes contained, in 1.5 ml.: 100 μmole phosphate (pH 7.5); 1 μmole MnCl₂; 100 μmole L-arginine hydrochloride; 8.3 and 8.7 mg. protein from wild type and mutant, respectively, added at time 0. After incubation at 35° for the times indicated, tubes were heated briefly and urea was degraded to ammonia by the addition of 0.5 ml. of urease solution.

In one experiment the mutant 36703, which resembles B317 etc. in requiring arginine and failing to respond to citrulline, but which is the result of mutation in a different chromosome (Newmeyer, 1957), was found to produce about the same amount of argininosuccinase activity as the wild type.

Other enzymes in mutant and wild-type strains

The wild type and the mutant B362 produced rather similar amounts of arginase and fumarase (Figs. 6, 7). Thus the elimination of argininosuccinase in the mutant was not accompanied by any gross effect on other enzymes related to argininosuccinase in substrate specificity.

DISCUSSION

The data on the accumulation of citrulline and argininosuccinic acid by the mutants when supplied with citrulline are consistent with the hypothesis of a complete 'block' in the conversion of argininosuccinic acid to arginine. It

seems probable that, when supplied with citrulline, the mutants convert it to argininosuccinic acid by combination with aspartic acid until the supply of the latter substance becomes limiting (probably through the observed depletion of the free amino-group pool of the mycelium), after which citrulline is accumulated as such. These effects of citrulline may account for its partial inhibition of growth of the mutants. The linear dependence of mutant growth on arginine supply at low arginine concentrations (Fig. 1) is also consistent with a complete inability to synthesize this substance. The observation that wild-type mycelium produces an active argininosuccinase which is apparently lacking in the mutants greatly strengthens the interpretation. There can be little doubt that argininosuccinic acid is a normal intermediate in arginine synthesis in *Neurospora* in spite of its failure to support growth of strain 36703, which produces argininosuccinase. It is probable that argininosuccinic acid supplied in the medium fails to penetrate the cell (cf. Newmeyer, 1957).

This investigation appears to add to the growing number of cases both in *Escherichia coli* (Davis, 1955) and in *Neurospora crassa* (e.g. Yanofsky, 1952; Horowitz & Fling, 1953; Fincham, 1954), where a mutation has resulted in the loss or alteration of a single enzyme. To this extent, the one gene-one enzyme hypothesis may be said to be receiving increasing support. However, although in the present study four mutants appeared to be biochemically indistinguishable, and all lacked the same enzyme, the genetic results given in the accompanying paper by Newmeyer (1957) suggest that they resulted from mutation at at least two distinct sites. B370 gave some wild-type ascospores in crosses with each of the other three mutants, and in the case of the B370 \times B317 cross it was shown that these wild types probably arose by crossing-over between two closely-linked loci. Thus the genetic basis of an enzyme may be a short chromosome segment, or complex locus, rather than a single gene in the sense of an indivisible unit.

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Arginine Synthesis in *Neurospora crassa*; Genetic Studies

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SUMMARY: Nine mutants of *Neurospora crassa* which require arginine as a nutrient but cannot use citrulline were obtained from various sources. These fall into two classes, according to location in linkage group I or VII. (Enzymic tests, reported elsewhere, indicate that the two classes of mutants affect the enzymes which control the two reactions between citrulline and arginine.) Heterokaryon tests between mutants of the same group were negative, and crosses between mutants of the same group were semi-sterile, most of the ascospores being non-viable. Crosses between the five group I mutants produced no *arg*⁺ progeny, and separate mapping tests on four of them indicate that they are either allelic or closely linked. All crosses between the group VII mutants gave many *arg*⁺ progeny. For the one pair of mutants which was studied in detail, origin of the *arg*⁺ by means other than crossing-over (or gene conversion) has been virtually eliminated. However, mapping studies place the two mutants only 0–6 units apart. It is concluded that the high *arg*⁺ frequency is due to selection, and that the mutants might be pseudoalleles.

In recent years several enzymic studies have been made in order to test directly the hypothesis that there is a one-to-one relationship between genes and enzymes (Beadle, 1945). Unfortunately, relatively few of these studies have been made on organisms in which it could be established that the mutant phenotype was really due to a single gene, and in these few cases there has usually been only one mutant available for study. A more critical test is provided when a series of similar mutants is studied; such tests are also needed to settle the controversy about whether pseudoalleles control the same or different reactions. However, in the case of biosynthetic enzymes, material suitable for such a test is so rare that very few cases of this sort have been reported (Yanofsky & Bonner, 1955; Fincham, 1950, 1954). The present study is an attempt to provide two more such cases.

The conversion of citrulline to arginine in mammalian liver has been shown by Ratner and co-workers (Ratner, Anslow & Petrack, 1953; Ratner & Petrack, 1953) to consist of at least two reactions:



The first step requires two enzymes, both of which must be present to produce any detectable effect. An enzymic study of wild type *Neurospora crassa* (Newmeyer, unpublished) showed that it converted citrulline to arginine in the same way as liver, except that there is no evidence yet that two enzymes are required for the first reaction. Independently, Drs Fincham & Boylen (personal communication) have obtained preliminary evidence for the same reaction sequence in *Neurospora crassa*, and they have studied the second reaction in detail (Fincham & Boylen, 1957).

This system seemed ideal for the type of test described above, because nine mutants were available which might be expected to fall into two or three genetic groups corresponding to the two or three enzymes involved. Furthermore, if pseudoalleles ever do control sequential reactions requiring close proximity of two gene-products (as discussed by Pontecorvo, 1955, and by Lewis, 1955), the conversion of citrulline to argininosuccinate should be an excellent place to look for it.

The present paper reports a genetic study of the nine arginine-requiring mutants. Each segregates as though a single mutant gene is involved. Genetically they fall into two classes, located in linkage groups I and VII; preliminary enzyme tests on one member of each class indicated that the group I mutant affects the first reaction and the group VII mutant affects the second (Newmeyer, unpublished).

The group VII mutants were sent to Drs Fincham & Boylen for study; their results, reported in the accompanying paper (Fincham & Boylen, 1957), indicate that all four mutants lack the enzyme which catalyses the second reaction. For their results to be most meaningful, it is necessary to know whether these mutations involve the same or different genes. Much of the present paper is devoted to an attempt to answer this question. Because of sterility difficulties, it has been necessary to restrict this work to two of the mutants; these appear to be either pseudoallelic or closely linked.

A similar study of the group I mutants is still in progress. Enzymic tests (Newmeyer, D. & Högström, D. unpublished) indicate that all members studied thus far affect the first reaction. The genetic tests to date, which are reported here, indicate that all five mutants may be alleles.

METHODS

Arginine-requiring mutants

Origin. Only two mutants of *Neurospora crassa* which can use arginine but not citrulline have been reported previously; these are 36703T (*arg-1*) and 46004, described by Srb & Horowitz (1944) and Srb (1946). Seven others were obtained by screening new mutants from various sources. Mutant H 4250 was kindly provided by Dr F. P. Hungate, who isolated it after treatment with ³⁵S (Hungate & Mannell, 1952). Particular thanks are due to Dr V. W. Woodward, who supplied seventy-two unclassified arginine mutants for testing, of which six were the desired type. Of these, mutants B 312 and B 317 were isolated after ultraviolet treatment, and mutants B 362, B 368, B 369, and B 370 after gamma-irradiation. The method for obtaining the last four mutants was such that two or more of them might possibly have arisen from a single mutated nucleus; however, the data reported here show that mutant B 369 is genetically different, and suggest that the other three are independent also. Examination of a total of nineteen additional arginine mutants, kindly provided by Mr R. W. Colburn, by Dr C. M. Stevens, and by Dr R. W. Barratt, yielded none of the desired type.

Genetic basis. The evidence that the arginine requirement of a given mutant segregates as a single Mendelian factor is based on the ratio of *arg*⁺:*arg*⁻

random isolates in crosses to wild type (or to other *arg*⁺ strains). For each mutant, Table 1 gives the pooled results of all such crosses in which the degree of germination was high enough ($\geq 70\%$) to minimize skewing of the results. Eight of the mutants gave the expected 1:1 ratio. In the case of the ninth mutant (B312), it is concluded that the deviation is due only to differential viability, for the following reasons: (1) the number of non-germinants is sufficient to account for the deviation; (2) if the ratio obtained were due to the segregation of two or more genes, then the cross B312 \times 30300 (Table 4) should give a minimum of 10% *arg*[±] progeny; actually it gave 1.0–1.5% *arg*⁺.

Table 1. *Segregation ratios*

Mutant	No. of random isolates		
	<i>arg</i> ⁺	<i>arg</i> [−]	Not germinated
B312	106	72	30
H4250	65	63	c. 20
46004	114	119	29
36703T	171	168	35
B369	62	56	12
B317	143	155	56
B368	52	72	29
B362	49	36	20
B370	244	265	79

Pooled data from various crosses. *P* values for deviations from 1 *arg*⁺:1 *arg*[−] are greater than 0.05, except for B312 where *P* = c. 0.01.

So far, all but two of the mutants appear to be point mutations. Mutant 36703T is associated with a translocation (Singleton, 1948), and shows linkage to groups I and V (Srb, cited in Singleton, 1948). There is also strong evidence that mutant H4250 is associated with some kind of chromosomal aberration (Newmeyer, unpublished). There is no evidence that the arginine requirement of either strain is separable from the aberration. The initial stock of mutant B362 also behaved aberrantly, but this defect was evidently eliminated in the first generation of inbreeding; only apparently normal B362 stocks have been used for enzyme studies or for the crosses reported here.

Inbreeding. All nine mutants were repeatedly back-crossed to wild *Neurospora crassa*, so that any enzymic differences found could more validly be ascribed to the arginine loci under study, rather than to other differences between the stocks. The wild type *Neurospora crassa* used for this purpose was STA (also called ST-74A), which was kindly provided by Dr P. St Lawrence; this strain was also used as the wild type for enzyme experiments, and was the parent of the six mutants isolated by Dr V. W. Woodward. In each generation an *arg*[−] *a* isolate was selected and crossed to STA; in the final generation of group I mutants, some *arg*[−] *A* isolates were also chosen, in order to make more isogenic the short region between *arg* and *sex*. All group I mutants were back-crossed for four to seven generations, and all group VII mutants for two to four generations, before being used for enzyme studies.

Relation to other arginine mutants. It will be shown that the strict arginine mutants studied by Srb (1946) are both in group I; the group VII mutants

apparently represent a previously undescribed locus (or loci). The standard group VII mutant, B317, is not allelic with the unmapped arginine mutants 34105, 21502, 35401 and 44297 (kindly supplied by Mrs M. B. Mitchell). All crosses were highly fertile and produced many wild type progeny. These tests were made even though the unmapped mutants can use citrulline or other precursors, because Mitchell & Mitchell (1952) showed that the ability of arginine mutants to use precursors is subject to modification by other genes. For similar reasons, the group I mutant 46004 was tested for allelism with 27947 (ornithineless), which was known to be near centromere in either group I or group II (see Barrett, Newmeyer, Perkins & Garnjobst, 1954); they segregated independently.

Culture methods and markers

Most of the arginine mutants studied will not grow well on standard complete media unless extra arginine is added; ordinarily 0.5 mg. L-arginine HCl/ml. was added to medium No. 2 of Tatum, Barratt, Fries & Bonner (1950), or modifications thereof. For crosses carrying the marker *button* (see below), better germination was obtained on supplemented minimal medium. Crosses were made on 'synthetic crossing medium' (SC) of Westergaard & Mitchell (1947), usually containing 0.1 mg. L-arginine HCl/ml. Sorbose minimal medium (to induce colonial growth) was used as previously described (Newmeyer, 1954), except that it usually contained only 0.5% sorbose and 0.1% sucrose. The minimal medium and standard genetic methods are described by Beadle & Tatum (1945); special methods will be described in connexion with specific experiments.

Genetic nomenclature and references to previously described markers are given in Barratt *et al.* (1954). The new colonial marker, *button* (*bn*, isolation number B40), was kindly supplied by Dr D. D. Perkins, who located it in linkage group VII; it was initially isolated by Dr V. W. Woodward.

RESULTS

Separation of the mutants into two classes

Classification according to growth on argininosuccinate (the intermediate between citrulline and arginine) was not possible, because *Neurospora* is relatively impermeable to this compound (Newmeyer, unpublished), as is *Escherichia coli* (Walker & Myers, 1953). However, the nine mutants were readily separated into two classes by linkage, heterokaryon and fertility tests.

The first class, consisting of mutants 36703T, 46004, B312, B369 and H4250, shows linkage to *sex* (in group I), in agreement with Srb's data for mutant 36703T. Members of the second class (mutants B317, B362, B368, and B370) show no significant sex-linkage, but are all linked to markers in group VII (the latter class of mutants was first located by Dr D. D. Perkins). Linkage data for both classes are summarized in Table 2. Further evidence for location in group I or VII is given below under *Allelism tests* for all mutants except B362 and B368; these two were not studied in detail because of the possibility that they arose from the same mutation as B370.

Heterokaryon tests confirmed the above division of the mutants, and gave no evidence for further subdivision of either group. All possible combinations of either two group I mutants or two group VII mutants gave negligible growth on slopes of minimal medium, but the combination of mutant B317 (group VII) with any group I mutant gave a definite positive test, as did the combination of mutant B369 (group I) with any group VII mutant. In these tests it seems unlikely that the negative reactions were due to incompatible

Table 2. *Data locating arginine mutants in linkage groups I and VII*

	Arginine mutant	Group I markers			Group VII markers	
		<i>sex</i> *	36703T	46004	<i>bn</i>	<i>nt</i> †
Group I	36703T	76P:8R	—	—	—	—
	46004	232P:17R	—	—	—	—
	B369	22P:3R	—	—	—	—
	B312	39P:2R	—	—	—	—
		(5 not scored)				
	H4250	57P:10R	—	—	—	—
		(+9 bisexual)				
Group VII	B317	7P:111R	5PD:3T:3NPD (41 % recomb.)	36 <i>arg</i> ⁻ :17 <i>arg</i> ⁺ (64 % recomb.)	36P:16R	49P:3R
	B370	19P:14R	—	55 <i>arg</i> ⁻ :16 <i>arg</i> ⁺ (45 % recomb.)	43P:16R	67P:1R
	B368	21P:16R	—	—	46P:10R	—
	B362	8P:16R	—	—	21P:4R‡	—

P=Parental random isolates; R=recombinant random isolates; PD=parental ditype asci; T=tetratype asci; NPD=non-parental ditype asci.

* Pooled data from various crosses. Data are not included if *sex* was scored only in a non-random sample of the isolates, in such a way as might introduce bias.

† Data of D. D. Perkins.

‡ Only 51 % germination. Germination in all other crosses was good.

combinations of heterokaryon-formation (*het*) genes, rather than to failure of the arginine mutants to complement each other. Garnjobst (1955) showed that, at least in the cases she studied, stable heterokaryons were formed only when the *het* genes of both strains were alike. On the assumption that all *het* genes involved here behave in this way, the isolates used for the present tests were all chosen from at least third generation backcrosses to wild type STA, to increase their chances of similarity, and a single isolate of each mutant was used for all tests. Since all mutants of one linkage group gave a positive test with the same isolate of the other group, it seems likely that they were all mutually compatible.

Failure to complement each other has been definitely demonstrated in the critical case of the group VII mutants B317 and B370, by isolation of the heterokaryon-compatible double mutants B370 *pan A* and B317 *nt A*. These were tested in growth tubes according to Ryan, Beadle & Tatum (1943). Neither strain alone grew in the absence of either of its required nutritional supplements; together they grew at wild type rate in each of three tests when only arginine was supplied, but failed to grow in each of three tests when only pantothenate and nicotinamide were present. The experiment was repeated on

slopes of solid media with similar results. Thus a clearly compatible combination of mutants B370 and B317 was unable to form an *arg*⁺ heterokaryon.

The classification of mutants described above is confirmed by fertility tests. Crosses between any two mutants of the same group have invariably been semi-sterile. On the best medium found (SC + 0.1 mg. L-arginine HCl/ml.) they formed abundant black perithecia, but ascospore production was low and the vast majority of spores were white and did not germinate. On the other hand, each of the nine mutants was highly fertile when crossed to any other stock tested. Crosses of group I by group VII mutants were also highly fertile in the four combinations tested.

Allelism tests

From the heterokaryon tests it might be presumed that the members of each of the two groups of arginine mutants are alleles. The near-sterility in intra-group crosses would point to the same conclusion, since sterility in crosses between apparent alleles is a frequent occurrence (see, for example, Mitchell & Mitchell, 1956; Pritchard, 1955). However, it is probable that both these types of evidence are essentially indications of a biochemical similarity, and, in a study designed to test the one gene-one enzyme hypothesis, decisions on allelism must be based on purely genetic criteria.

Methods. The near-sterility of crosses between mutants of the same group creates a major obstacle to obtaining direct genetic evidence on allelism. Many attempts were made to overcome this problem, with some success. A major cause of the sterility is evidently that the high arginine concentration required for optimal growth (0.5–1.0 mg. L-arginine HCl/ml.) provides a higher concentration of available nitrogen than is compatible with the production of functional perithecia. The inhibition of perithecium formation by high nitrogen concentrations was described by Hirsch (1954). The best solution to this problem found so far is to make crosses in Petri plates on SC medium (Westergaard & Mitchell, 1947) containing a suboptimal concentration of arginine, and to add further arginine supplements after fertilization. In practice, 15 ml. of medium, usually containing 0.1 mg. arginine/ml., was used per plate. The cross was fertilized at 6–7 days, and six or more additions of sterile arginine solution (usually 0.15 ml. of a 10 mg./ml. solution) were made at a single point at the edge of the plate, usually at daily intervals, beginning 1 or 2 days after fertilization. Most of the ripe perithecia appeared in a zone $\frac{1}{2}$ – $1\frac{1}{2}$ in. from the point of addition, and shot their spores on to the lid of the plate.

This procedure gave a great increase in ascospore production, but little or no improvement in the proportion of ripe spores. As many spores as possible were therefore scraped from the lid, freed from any mycelium growing there, heat-shocked, and plated in sorbose medium to screen for the few which were viable. Initial experiments were done by selective plating, i.e. spores were plated with and without arginine, and the % of *arg*⁺ mutants estimated from the numbers of colonies on the two types of media. To obtain more accurate values, some experiments were done by 'total isolation', i.e. all spores were plated with arginine, and all adequately separated colonies isolated and tested.

The same plates were sometimes used both for total isolation and for the estimated viable count on a selective plating.

Approximate information about allelism may also be obtained indirectly by mapping, to determine whether all mutants are in the same region. The results of both methods are given below.

Group I mutants. The direct-crossing method was less successful for group I mutants than for those in group VII. The sparse results for group I are given in Table 3; since no *arg*⁺ colonies were found, it is concluded that mutants 46004, H4250, B312, and B369 must at least be closely linked. Crosses B, C, D and F were analysed as described above. For cross A, the spores were instead heat-shocked on plates of complete medium without sorbose, and the few which germinated isolated and tested. For cross E, single ripe-looking spores were isolated directly. These two methods are too slow to be generally useful.

Table 3. *Crosses between group I arginine mutants*

Cross	Parents	No. of viable spores	No. of <i>arg</i> ⁺ spores	Method
A	46004 × H4250	176	0	Isolation of germinated ascospores
B	46004 × H4250	27*	0	Selective plating
C	46004 × 36703T	31*	0	Selective plating
D	46004 × B312	452*	0	Selective plating
E	46004 × B312	38	0	Random isolates
F	46004 × B369	148*	0	Selective plating

* Estimated.

Because these mutants are located in a well-marked chromosome region, all further study of them was done by mapping. Various crosses of mutant 46004 to known markers showed that it is located between *sex* and *crisp*, and very close to *ad-5*. This location suggested close linkage to the marker *arg-3* (30300), which can use either citrulline or arginine. Ascospores from a cross of mutants 30300 × 46004, plated on sorbose medium, gave only one definite and one questionable *arg*⁺ colony out of approximately 665 viable spores. To eliminate spurious wild types arising by heterokaryon formation, ascospores from crosses of 30300 × 46004 and of 30300 by three other group I mutants were then plated on minimal agar and scored as *arg*⁺ or *arg*⁻ by the amount of growth produced shortly after germination, as described by Mitchell, Pittenger & Mitchell (1952). The rare apparent wild types, both in and out of the counted area, were isolated and retested, and those which proved to be really *arg*⁺ were scored for *sex* to obtain information on gene order. Of nineteen putative *arg*⁺ which were isolated and retested, two were actually *arg*⁻; therefore any *arg*⁺ which could not be isolated, because of closeness to other spores, were recorded as 'uncertain'. Results to date are summarized in Table 4. They indicate that all four arginine mutations are located within three map units of 30300, and probably between 30300 and *sex*. It is not yet known whether the lower *arg*⁺ frequency found for the 30300 × 46004 cross is valid. This cross is more difficult to score,

so that in this (but not in the other three crosses) occasional *arg*⁺ might go undetected. A cross of mutant 30300 by the fifth group I mutant, H4250, was not scorable by this method.

Table 4. *Crosses of 30300 (citrulline) × group I arginine mutants*

Ascospores classified by growth after germination on minimal agar plates.

Cross	No. of <i>arg</i> ⁺ spores in counted areas		Total germinated spores in counted area	% <i>arg</i> ⁺	Sex of certain <i>arg</i> ⁺ (not restricted to counted area)
	Certain	Uncertain			
30300 a × 46004 A	2	1*	c. 4300	0.05–0.07	2a, 0A
30300 a × B312A‡	4	2†	409	1.0–1.5	4a, 1A
30300 a × B369A‡	3	5*	611	0.5–1.3	7a, 1A
30300 a × 36703T A	2	1*	468	0.4–0.6	2a, 0A

* Could not be isolated and retested.

† Possibly due to heterokaryon formation.

‡ 5 certain *arg*⁺ from these crosses were crossed to wild type and found to breed true.

Group VII mutants. The first crosses between mutants in this group gave an unexpected result: many *arg*⁺ progeny were obtained from each combination tested; namely, B370 × B362, B370 × B368, and B370 × B317 (Table 6, first three lines). On the face of it, this would mean that the enzyme argininosuccinase was controlled by widely separated loci. However, in view of the data in Table 2 (which indicate rather close linkage), and also of the possible single origin of strains B362, B368 and B370, this explanation seemed so unlikely that it was suspected that the arginine-independent colonies arose from some cause other than crossing over, e.g. reversion or contamination, or that they were really only pseudo-wild types, as described by Mitchell *et al.* (1952).

Because of the great labour required to produce enough ripe spores to settle this question, further *arg*[−] × *arg*[−] crosses were confined to the single combination of strains B370 × B317. At the same time, careful mapping of each of these mutants was begun; the results showed clearly that B370 and B317 were at most a few units apart, and might well be alleles. The results of the *arg*[−] × *arg*[−] crosses showed almost equally clearly that the *arg*⁺ progeny were nevertheless true recombinants; their high frequency apparently is due to selective ripening of ascospores. The two mutants must therefore be either pseudoallelic or closely linked. The evidence is as follows.

The mapping consisted of a series of three-point crosses, each involving an arginine mutant plus the two closest markers, *bn* and *nt*. The results are given in Table 5. It is clear that for both arginine mutants the order is *bn*--*arg*--*nt*. In one cross the distance *arg* (B316)--*nt* is somewhat greater than the average value for *arg* (B370)--*nt*, but it is questionable whether this difference is real, considering the wide variation between crosses. Similar variations between crosses in different genetic backgrounds have been noted in *Neurospora* by Stadler (1956), and by F. de Serres, R. W. Barratt, and others (personal com-

munications). In any case B370 and B317 are most probably between 0 and 6 units apart.

Table 5. *Group VII three-point crosses*

Numbers of random isolates from crosses involving the markers button (B40), arginine (either B317 or B370), and nicotinic acid+tryptophan (65001).

Parent genotypes	Parental combinations	Recombinations		
		Singles region 1 (<i>bn-arg</i>)	Singles, region 2 (<i>arg-nt</i>)	Doubles, regions 1 and 2
$\frac{+ arg\ nt}{bn\ +\ +}$ (<i>arg</i> =B370)	43 + - -	7 + + +	2 + - +	0 + + -
	36 - + +	4 - - -	0 - + -	0 - - +
	79	11	2	
		(12.0 %)	(2.2 %)	
$\frac{+ arg\ nt}{bn\ +\ +}$ (<i>arg</i> =B317)	38 + - -	5 + + +	1 + - +	0 + + -
	37 - + +	6 - - -	0 - + -	0 - - +
	75	11	1	
		(12.6 %)	(1.2 %)	
$\frac{+ + +}{bn\ arg\ nt}$ (<i>arg</i> =B370)	79 + + +	22 + - -	1 + + -	1 + - +
	60 - - -	14 - + +	3 - - +	0 - + -
	139	36	4	1
		(20.0 %)	(2.2 %)	(0.6 %)
$\frac{+ arg\ +}{bn\ +\ nt}$ (<i>arg</i> =B370)	36 + - +	15 + + -	0 + - -	0 + + +
	29 - + -	9 - - +	2 - + +	0 - - -
	65	24	2	
		(26.4 %)	(2.2 %)	
$\frac{+ arg\ +}{bn\ +\ nt}$ (<i>arg</i> =B317)	31 + - +	8 + + -	4 + - -	0 + + +
	31 - + -	11 - - +	3 - + +	0 - - -
	63	19	7	
		(21.6 %)	(8.0 %)	

Results of the *arg*⁻ × *arg*⁻ crosses are given in Tables 6 and 7. All crosses involving two different arginine mutants, i.e. B370 × B317, will be called intercrossoes (IC), and all crosses homozygous for one arginine mutant, e.g. B370 × B370, will be called selfings (S), regardless of the distribution of the marker *nt*. The results make highly improbable all obvious origins of the *arg*⁺ progeny other than crossing over.

A high rate of reversion (or suppressor mutation) is eliminated by the absence of *arg*⁺ progeny in the selfings. Selfings appear to be even less fertile than intercrossoes; nevertheless, the few data it has been possible to obtain are sufficient to eliminate sampling error. *P* for homogeneity of either selfing with pooled intercrossoes, by χ^2 test on the 'total isolation' figures, is < 0.001 . All isolates from the selfings were tested twice to insure that the rare *arg*⁺ was not overlooked. It was also shown, by plating IC7 at different spore concentrations, that the absence of *arg*⁺ in the selfings was not due to any effect of population density on their survival. Tests for reversion during vegetative growth were also negative.

The results might still be explained by rare reversion, which by chance affected only the cultures used for the intercrossoes, followed by selection for the *arg*⁺ progeny. This explanation seems unlikely because several different isolates

Table 6. *Crosses between group VII arginine mutants*

Cross number	Parents	Total isolation				Selective plating		
		Total colonies	Total isolated	No. of <i>arg</i> ⁺ isolated	% <i>arg</i> ⁺ among those isolated	Estimated total viable	No. of <i>arg</i> ⁺	<i>arg</i> ⁺ isolate (c. %)
IC1	B370 × B362	—	—	—	—	400	37	9
IC2	B370 × B368	—	—	—	—	490	11	2
IC3	B370 × B317	—	—	—	—	196	25	13
IC4	B370 × B317	—	—	—	—	102	17	17
IC5	B317 × B370 <i>nt</i>	6	4	1	25.0	21	7	33
IC6	B370 × B317	497	212	93	43.8	—	—	—
IC7	B317 × B370 <i>nt</i>	62	41	17	41.5	—	—	—
IC8	B370 × B317 <i>nt</i>	29	27	17	63.0	29	11	38
IC9	B370 × B317 <i>nt</i>	10	10	4	40.0	8	6	75
S1	B370 × B370	9	9	0	0	—	—	—
S2	B370 × B370 <i>nt</i>	44	38	0	0	—	—	—
S3	B317 × B317 <i>nt</i>	36	32	0	0	18	0	0
S4	B317 <i>nt</i> × B317 <i>nt</i>	6	6	0	0	—	—	—

Selective plating gives only a rough estimate of the % *arg*⁺ isolates when the number of viable spores is so small, particularly since the viable count must be based on very few plates. Total isolation is not subject to these errors.

In IC7, 8 and 9, a dark ascospore could be seen in 52 of the 78 colonies isolated; 50.0 % of those with visible dark spores were *arg*⁺, compared to 48.7 % of the total.

For crosses carrying *nt* (65001), excess nicotinamide was added to all plates.

All spores were obtained by the plate-crossing method. The yield of viable spores per cross-plate varied from 5 to 22 for selfings and from 14 to 124 for intercrosses made under comparable conditions.

were used, and because both isolates used as parents for IC7 were also used as parents for S2 and S3.

Contamination during plating of ascospores is excluded because most colonies could be seen microscopically to have arisen from a dark ascospore. For IC7, 8, and 9, the numbers of colonies in which the spore could be seen were counted separately (Table 6); they yielded approximately the same *arg*⁺ frequency as did the total data.

Contamination during ascospore formation seems very unlikely, because of the high reproducibility of the results, and because intercrosses heterozygous for *nt* always gave some *arg*⁺*nt*⁻ progeny (Table 7).

Tests for pseudo-wild types were carried out on eleven representative *arg*⁺ isolates from the B370 × B317 crosses, including two *arg*⁺*nt*⁻ from colonies with visible dark ascospores. These were crossed to standard wild types and usually tested by isolating 50 spores at random, since the group VII arginine mutants are hard to score on minimal agar plates. No *arg*⁻ progeny were found in any case. Therefore the 11 *arg*⁺ tested are not pseudo-wild types, and on statistical grounds it is likely that the same holds for most of those not tested.

Heterokaryon formation is eliminated by the double-mutant heterokaryon tests described earlier, and also by the pseudo-wild type tests. Non-genetic adaptation is excluded by the absence of *arg*⁺ progeny in the selfings, and by the pseudo-wild type tests.

Thus all obvious origins of the *arg*⁺ progeny other than recombination are virtually eliminated. Recombination is also indicated by the data on the segregation of *nt* (Table 7). However, the true recombination frequency can not be reflected by the frequency of the *arg*⁺ progeny, since this would require an unprecedented 90 % recombination, which is in any case excluded by the three-point cross data. The only simple explanation would seem to be selection.

Table 7. Segregation of *nt* in group VII intercrossoes

All adequately separated *arg*⁺ colonies from selective platings were isolated, and their results added to those from the total isolations; the results obtained by the two methods were homogeneous in all cases. The *arg*⁻ classes are included to show that the skewing in the *arg*⁺ classes is not due to selection for or against *nt*⁻.

	B370 × B317 <i>nt</i>			B370 <i>nt</i> × B317		
	IC8*	IC9*	Total	IC7*	IC5†	Total
<i>arg</i> ⁺ <i>nt</i> ⁺	20	4	24	3	2	5
<i>arg</i> ⁺ <i>nt</i> ⁻	4	6	10	14	6	20
<i>arg</i> ⁻ <i>nt</i> ⁺	4	2	6	16	0	16
<i>arg</i> ⁻ <i>nt</i> ⁻	6	4	10	8	3	11

* *nt*⁻ was carried by the fertilizing parent.

† *nt*⁻ was carried by the protoperithecial parent.

Indeed, there is reason to believe that the near-sterility of these crosses is largely due to a deficiency of arginine inside the perithecia. If, because of this deficit, only *arg*⁺ spores and their closest *arg*⁻ neighbours were able to ripen, there might be intense selection for *arg*⁺. It is hard to make an accurate estimate of ascospore viability because the unripe spores are nearly transparent; a very rough count for the cross IC6 indicated that only about 0.067 % of the shot ascospores survived. If all the non-viable spores were *arg*⁻, the true *arg*⁺ frequency would be only 0.023 %; i.e. within the range for crossing over between pseudoalleles.

Since the *arg*⁺ isolates evidently do arise by recombination, it is of interest to know whether the process results in restoration of the wild-type enzyme, and whether any trace of the arginine requirement remains. Drs Fincham & Boylen (personal communication) have tested six representative *arg*⁺ isolates from the B370 × B317 crosses, including two *arg*⁺*nt*⁻ which had been tested for pseudo-wild types. All of them had argininosuccinase activity like that of the standard wild type. Also, three *arg*⁺*nt*⁺ isolates were tested in flask cultures. Dry-weight determinations showed that arginine was not significantly more stimulatory for these isolates than for the standard wild-type controls.

DISCUSSION

The sterility problem has prevented localization of either group of mutants to an interval of less than a few map units. For the group VII mutants it obviously is not feasible to go further until better markers or a better crossing method become available; indeed, the analysis would never have been continued this far, had it not been necessary in order to give meaning to the

enzymic studies. The group I mutants, on the other hand, warrant further study, since an abundance of close markers partly compensates for the sterility of intra-group crosses.

Despite the technical difficulties, this investigation has been fruitful in two respects. First, the group VII mutants provide a new case in which major control of a biosynthetic enzyme is apparently restricted to a small chromosome region, and it seems likely that the group I mutants will add still another case when enzymic tests are completed. Previous work on biosynthetic enzymes suggests that, although changes at various loci can cause minor changes in activity of the same enzyme (Hogness & Mitchell, 1954; Yanofsky & Bonner, 1955), there is only one locus at which mutation can cause a deficiency so severe that it results in a nutrient requirement (tryptophan synthetase: Yanofsky & Bonner, 1955; and Weijs, 1954; glutamic dehydrogenase: Fincham, 1950, 1954). In the case of catabolic enzymes on the other hand, it is clear that severe enzymic deficiencies can be caused by mutations at numerous widely separated loci (lactase: Lederberg, 1951; O. Landman, cited in Bonner, 1951; tyrosinase: Markert, 1950). It appears that many of these loci act by controlling the production of other proteins needed for the induction of the enzyme itself (Monod, 1956; Landman, cited in Bonner, 1951), and it is perhaps reasonable that catabolic enzymes should be more subject than biosynthetic ones to such multi-gene control. The question remains whether the distinction between the two types of enzyme has any validity, or whether it merely results from the very small number of biosynthetic enzymes for which more than one mutant has been tested. The present two cases support the idea that the distinction is real, even though neither group of arginine mutants is localized to as small a chromosome region as would be desirable.

This investigation also provides a second case, in addition to tryptophan synthetase, in which mutants which cause the loss of the same biosynthetic enzyme are apparently separable by infrequent crossing over. However, the recent work of Lindegren (1955) and Mitchell (1955) raises the possibility that the apparent recombinants are really due to 'gene conversion'. The data in Table 7 indicate an excess of apparent double cross-overs like that found in several studies of complex loci (e.g. Pritchard, 1955; Mitchell, 1955; St Lawrence, 1956; Giles, 1956). In Mitchell's case (but not in Pritchard's) these proved to be due to conversion rather than recombination. With present methods it seems impossible to eliminate conversion in the case of either argininosuccinase or tryptophan synthetase. Until it is eliminated in a case which can be studied enzymically, direct evidence that pseudoalleles can govern the same enzyme will be lacking.

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Some Studies on the Identification of Rumen Bacteria with Fluorescent Antibodies

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SUMMARY: Fluorescent antisera to a number of isolates of rumen bacteria have been prepared and used to demonstrate the presence of these organisms *in situ* in rumen contents. The serological tests for a specific organism in rumen contents of a number of calves agree with the isolations of this organism from the same sources. A microscope suitable for this work, the preparation and purification of antisera and the preparation of specimens is described.

Much work is being done at present on the biochemistry and bacteriology of the rumen contents of cattle and sheep. Two main methods of investigation are being used. One studies the reactions of whole, or almost whole, rumen contents transferred to suitable vessels *in vitro*, the other isolates organisms through the use of suitable media and then studies the reactions of these organisms in pure culture. In the latter case it is necessary to show that the organism which is being studied is one which is of importance in the rumen. There seems to be no general agreement among workers in this field about what is 'a true rumen organism'. Morphology alone is not conclusive as many rumen organisms have a similar appearance, and conversely, pleomorphism is common. In addition, an organism may not attack substances *in vitro* which it apparently attacks *in vivo*. This might be due to an unsuitable medium *in vitro*, or to the organisms being physiologically different, although having similar morphology. A method which shows the isolated organism near the foodstuff thought to be attacked would settle the problem. It would also be of interest to see whether similar organisms occurred in more than one animal or in different parts of the gut of the same animal without having to use isolation procedures. The presence of an organism in more than one animal may be an indication of its being a true rumen organism. These, and other considerations suggest that a method of identifying rumen organisms *in situ* would be of help. MacPherson & Oxford (1952) described some work on the serological identification of rumen streptococci using the Neufeld 'Quellung' reaction, but this reaction is difficult to see in rumen contents and in any case is applicable only to organisms with a large capsule. However, antisera can be labelled by dyes visible in white or ultraviolet (u.v.) light in such a way as to provide a visual indication of their combination with a specific antigen. Fluorescent dyes have been applied to the localization of antigens in tissue sections (e.g. Coons, Snyder, Cheever & Murray, 1950; Gitlin, Landing & Whipple, 1953; Humphrey, 1955), and Coons & Kaplan (1950) have described the preparation of fluorescein isocyanate and the coupling of this with serum proteins for such investigations. It was decided to try to apply this method to the identification *in situ* of

rumen bacteria, as the method should distinguish any bacteria which react with the labelled antiserum and not only those which possess large capsules. A preliminary note on the method has been published (Hobson, Mackay & Mann, 1955); further details are given below.

METHODS

Microscope. The microscope is shown diagrammatically in Fig. 1. It has been built up from parts of a number of microscopes, but most types could be adapted for use in similar work. A horizontal microscope was decided upon to allow the maximum intensity of u.v. radiation to enter the condenser and a monocular body gives a brighter image than binocular eyepieces. A powerful

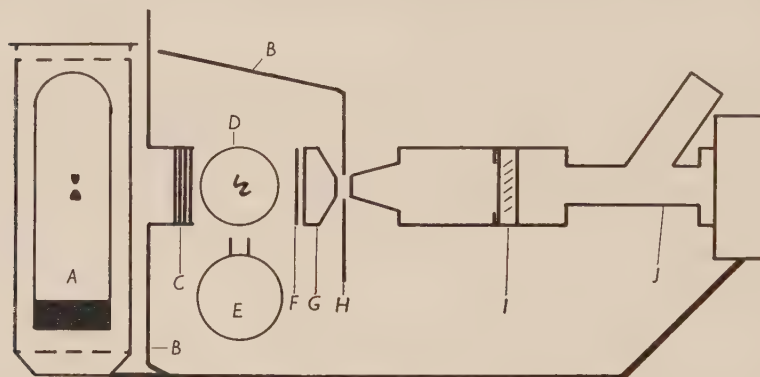


Fig. 1. *A*, High-pressure Hg arc with air cooling; *B*, screens; *C*, ultraviolet filter; 6 mm. Chance OX1 glass, 2 mm. Wood's glass; *D*, source of white light; *E*, mirror to direct white light into microscope; *F*, phase annulus; *G*, quartz condenser; *H*, microscope stage; *I*, sodium nitrite solution filter; *J*, 35 mm. camera.

source of u.v. radiation is needed and for this a 250-watt high-pressure mercury arc (Type ME/D, British Thomson-Houston Ltd., Rugby) is used. The lamp is fitted in a centering metal-housing with an air blast for cooling. Glass filters are adequate for cutting out most of the visible light and transmitting the longer u.v. wavelengths (about 3650 Å.) needed; the required thickness of filter was found by trial. A source of white light (in the following text white light is used to denote visible light used for examining objects, although blue or green filters were used as in ordinary microscopy) is placed at the side of the microscope and the mirror *E* can be swung up to direct this light into the condenser. When most of the pictures shown here had been taken it was found possible to convert the microscope for phase-contrast by fixing a phase annulus to the glass disk *F*, which can be swung into the white light beam and then focused on the phase plate of an oil-immersion objective. The quartz condenser is not strictly necessary for this work; an ordinary two lens Abbe condenser is adequate if it does not itself fluoresce. Normal glass objectives are used, the most useful being a $\times 90$ achromatic objective fitted with an adjustable stop. To protect the eyes 5 mm. of 10% (w/v) sodium nitrite solution in an optical

glass cell (made to fit on the tube diaphragm of the microscope by Tintometer Ltd., Salisbury, Wilts) is used. This sodium nitrite filter is preferred to glass filters as it does not change the colour of the fluorescent images, which is of great help in identifying some particles. The sodium nitrite solution is freshly made up and filtered when required; otherwise the cell is kept dry. A yellowish tinge develops on the glass after some months, but this can be removed with chromic + sulphuric acid. For routine observations a $\times 8$ or $\times 10$ eyepiece is used and for photography a 35 mm. camera and viewing eyepiece (Beck Ltd., Mortimer Street, London, W. 1) is attached. The whole apparatus is mounted on a rigid framework which passes through the screen. 'Fluoroil' (E. Gurr Ltd., 42 Upper Richmond Road, London, S.W. 14) was generally used as immersion fluid. All lenses and filters should be free from finger marks or dust as these cause undesirable blue fluorescence in ultraviolet radiation. The filters used allow u.v. radiation and some blue and red light through, but the red is almost entirely focused out when the condenser is properly set for u.v. radiation (the condenser needs to be re-focused for white light). A cardioid condenser has also been used; this was not much improvement for u.v. radiation over ordinary illumination and was a disadvantage in identifying objects in white light. The best conditions for observing the fluorescence are with the oil-immersion objective stopped down to about N.A. 1. Observations are made in a dark room.

Photography. Since the viewing eyepiece transmits only a portion of the light which enters the camera it is sometimes difficult to see the images of fluorescent objects. Scanning is therefore done without the camera. If phase contrast is not used for white light the condenser diaphragm must be very much closed down to give any image at all and this accounts for the long exposures needed and the defects of the filters apparent in the photographs shown. Micro-file panchromatic film (Kodak Ltd., London) was used for all but one of the photographs shown. The exposures for fluorescence were 5–20 min. and for white light about 10 sec. Faster films did not give as good enlargements.

Organisms. The isolation of most of the organisms used for preparing antisera has already been described, or will be described (MacPherson, 1953; Mann, Masson & Oxford, 1954; Mackay & Oxford, 1954; Mann & Oxford, 1954, 1955). All organisms were obtained from rumen or abomasum contents by the usual dilution techniques in suitable media.

Antisera were prepared in rabbits by the injection of washed formalin-killed or heat-killed suspensions of the organisms, over a course of some weeks. The titres were often quite low (streptococci 1/200 to 1/1000; lactobacilli 1/1000) but some of the Gram-negative cocci and Gram-negative rod antisera gave higher titres (1/10,000 to 1/20,000).

Preparation of aminofluorescein. This followed the method of Coons & Kaplan (1950) except that hydrogenation of the nitrofluorescein took about 9 hr. for each preparation and not $1\frac{1}{2}$ hr. as these authors stated. Aminofluorescein I was generally used for conjugation.

Conjugation of antisera with fluorescein isocyanate. In general the procedure

of Coons & Kaplan (1950) was used, but the phosgene was obtained from a cylinder (I.C.I. Ltd., Widnes, Lanes) and the apparatus was thus under slight positive pressure. A column of liquid paraffin, coloured by a little Sudan III, acted as manometer and safety valve. The *isocyanate* solution was added slowly to the stirred serum solution at 1° with an all-glass hypodermic syringe but even so, a small precipitate was formed. Some of this precipitate dissolved in the usual stirring-time of 20 hr., and continuation of stirring for 48 hr. did not lead to further dissolution. After dialysis the solutions were cleared by centrifugation at 20,000 *g* for 30 min. at 0°. The buffered saline referred to later was the same solution as that used in the conjugation of the sera, but filtered through a G5 glass filter.

Fractionation of antisera. After conjugation a globulin fraction was obtained by precipitation of the serum + saline solution (25 ml.) with 18 % (w/v) Na_2SO_4 at room temperature (Humphrey & MacFarlane, 1954). The precipitate was washed with 18 % (w/v) Na_2SO_4 and dissolved in sterile buffered saline (5 ml.). Paper electrophoresis showed that initially the serum contained two main protein components corresponding to the albumin and γ -globulin and both were fluorescent. The fractionated sera contained only one component, this was fluorescent and corresponded with the globulin of the original serum.

Preparation of protozoan powder. A suspension of rumen holotrich protozoa filled with starch granules was prepared by the method of Masson & Oxford (1951), and the organisms dehydrated by washing in acetone and finally dried *in vacuo* over P_2O_5 .

Preparation of mouse-liver powder. The livers of freshly killed mice were dissected out and chopped into physiological saline. After washing in saline the liver was ground to a fine powder in acetone and finally dried *in vacuo* over P_2O_5 .

Absorption of antisera. ('Serum' in this and later paragraphs means the final solution of serum in buffered saline obtained after conjugation and dialysis as above.) For absorption by liver powder, protozoa or starch, the serum was shaken with the powder (100 mg./ml.) at room temperature for 1 hr. and cleared by centrifugation. One absorption was usually sufficient. Absorption with bacteria was carried out by adding a thick suspension of the bacteria to the serum, shaking gently at room temperature for a few minutes and centrifuging. The reaction of the serum was tested under the microscope and when necessary the treatment was repeated.

Storage of antisera. The conjugated sera were stored at 2° without additions. The whole unabsorbed conjugated sera have been kept for periods of up to two years, but after this time they seem to lose some of their specificity and give a poor fluorescence. After a few months storage, a slight cloudiness generally appeared in the sera, but this could be removed by centrifugation and it did not affect the reactions of the antisera. Occasionally a contaminant organism grew in samples of sera but, again, they could be cleared by centrifugation. Attempts to filter the sera were not successful.

Preparation of test slides. Thin (0.8 mm.) glass slides were initially used but better results were obtained using u.v. glass slides (Reicherts, Vienna).

Ordinary coverslips were used. Samples of rumen contents were obtained either through permanent fistulae or by stomach tube, and of abomasum contents from the slaughter house. The samples were in most cases strained through gauze to remove the coarser particles. For rapid tests a sample of the rumen contents (1 loop) was mixed with buffered saline (1 loop) and antiserum (1 loop) on the slide, covered, blotted and examined immediately. Rather better results were obtained when the rumen contents (1 vol.) were mixed with buffered saline (2 vol.) and serum (0.5–1 vol.) in a tube and incubated at 35° for 30 min.; each volume need be only 0.1 or 0.2 ml. The buffered saline kept the pH value at about 7 (the optimum for fluorescein fluorescence), but with calf abomasum contents of initial pH value about 2, a more concentrated buffer was needed. Drops were removed from the tubes for examination as wet films.

In general it was found that the following points must be noted in preparing slides. Loops, pipettes, etc., used in the antisera should not be warm, as hot apparatus coagulates some serum proteins and gives indefinite fluorescent masses on the slide. The films should be pressed as thin as possible to obviate background fluorescence from the layer of antiserum, but bubbles must be avoided or the slide will appear to have streaks and blobs of fluorescent material around the edges of, and in, the pressed-out bubbles.

RESULTS

Tests with different organisms showed that the antisera did not change their specificity on conjugation with aminofluorescein. With all the organisms tested one cell combining with the antiserum can easily be seen, but the degree of fluorescence varies amongst the organisms, the brightest being found, as expected, in the capsulated streptococci. Addition of non-conjugated homologous antiserum before adding conjugated antiserum prevents green fluorescence of the organisms. Incubation for 30 min. is sufficient to obtain maximum fluorescence. The organisms alone are generally black or faint blue in u.v. radiation with a suggestion that organisms grown in agar-containing media have a slightly increased blue fluorescence.

Naturally-occurring fluorescing materials in rumen contents are usually blue or white and occasionally red or yellowish, and objects which absorb conjugated antiserum fluoresce green. When the rumen contents are not initially strained through gauze or silk, the coarser particles of hay, etc., make the films thick and give a green fluorescent background, making it difficult to pick out fluorescing bacteria. In the initial tests with untreated unabsorbed conjugated antisera it was found that protozoal and foodstuff starches took up some serum. This non-specific reaction was not materially removed by absorption of the sera with dried protozoa or whole potato starch. This fluorescence could, however, be overcome by allowing the antisera to react with the rumen contents, then centrifuging the whole suspension at high speed and, after removing the supernatant fluid, washing the sediment with buffered saline. The sediment was then resuspended in buffered saline and films made as usual. This method had the disadvantage that all the foodstuff particles and organisms

were compacted into one mass and no conclusions could be drawn as to the original positions of the organisms in relation to the foodstuffs; it was not generally used. Films of rumen contents fixed by heat or formalin appeared to absorb serum in a non-specific manner even after washing; again the original site of the organisms with relation to food particles, etc., was uncertain, so this method was not further investigated. Precipitation of a γ -globulin fraction decreased the non-specific reactions, but absorption of the antisera or globulins with liver powder gave the best results. The absorbed antisera seem to be quite specific in their reactions and normal rabbit serum conjugated with fluorescein and absorbed did not react with anything in rumen contents (Pl. 1, figs. 1 and 2).

To enable a better picture to be obtained in white light attempts were made to stain the organisms in wet films with dyes as well as with conjugated antisera. Although a number of common bacterial stains were tried, in all cases it was found that sufficient stain to make the bacteria easily visible in white light masked or prevented the fluorescence of organisms treated with antiserum. The results of tests with a number of bacteria are given below. Specific organisms added to rumen contents could easily be picked out by using the conjugated antisera.

Amyolytic streptococci (related to *Streptococcus bovis*) have been isolated from sheep and calves, and serological tests have shown that many of these isolates are similar and are widely distributed. Plate 1, fig. 3 shows a pure culture of one strain of these organisms reacting with conjugated antiserum, and Pl. 1, figs. 5 and 6, show a similar organism in a mass of material from sheep rumen contents reacting specifically with a conjugated antiserum. In these organisms the type specific antigen is in the capsule (Hobson & MacPherson, 1954) and this fluoresces green, the cell body being darker.

Two different species of Gram-negative cocci have so far been isolated, one a facultative anaerobic coccus, *Sarcina bakeri* (Mann *et al.* 1954) from the sheep, and an anaerobic coccus, from the calf rumen, details of which are to be published (Mann & Oxford). This calf-rumen coccus is probably related to the sheep-rumen coccus 'LC' described by Elsdon *et al.* (1956). Both cocci vary in morphology and also in apparent reaction with antiserum. Some organisms appear to react only in a thin surface layer, the cell body being dark, with some the whole organism appears fluorescent and some *S. bakeri* organisms do not react. After absorption by liver powder the antisera to the two organisms did not cross-react, but some cross-reaction was found between two isolates of the anaerobic coccus, although the sera were specific at high dilutions. This cross-reaction was removed by absorbing one antiserum with the other organism. Investigations so far have shown that the Gram-negative cocci fluctuate in numbers in any one sheep or calf. Plate 2, figs. 7 and 8, shows some of the *S. bakeri* in chain formation reacting with specific antiserum. In this case it will be noted that two of the organisms at the end of the chain had apparently not reacted with the antiserum. This phenomenon has been noted in pure cultures. Plate 2, figs. 9 and 10, shows one serological type of the anaerobic coccus reacting with antiserum in calf rumen contents. Experiments with six calves showed that where these organisms could easily be picked out in

samples of rumen contents tested with conjugated antisera they could be cultured from the rumen contents in numbers equivalent to *c.* 10^{10} /ml. Where the organisms were not found by the serological tests they were either absent or isolated only at concentrations equivalent to 10^2 or 10^4 /ml. rumen contents. Further work is in progress on these organisms, but in both groups there seem to be a number of different serological types present in the rumen.

A number of species of lactobacilli, including *Lactobacillus brevis*, *L. fermenti* and *L. acidophilus*, were isolated from calves and conjugated antiserum to a mixture of antigens of some of these isolates was prepared. Plate 1, fig. 4, shows lactobacilli in mixed culture reacting with this antiserum and revealed by fluorescence under u.v. irradiation. Tests with rumen and abomasum contents from calves 7 and 24 days old receiving a milk diet showed the presence of lactobacilli which reacted with the antiserum, but many of these organisms were irregular in shape and somewhat granular. In one slide from the rumen contents of the first calf there seemed to be a definite cross-reaction of this antiserum with a group of small diplococci. Lactobacilli were not found in the rumen contents of calves or of a sheep fed on the normal adult animal diet. Many of the slides of gut contents of the younger animals were blurred by coagulated milk particles.

The isolation from the rumen of Gram-negative rods, mainly coliform types, has been described (Mackay & Oxford, 1954) and conjugated antisera to these were prepared. The organisms are very pleomorphic, especially in older cultures, and the antiserum usually seems to react with a thin 'slime' layer around the organisms. A number of these organisms in sheep rumen contents which have reacted with conjugated antiserum are shown in Pl. 2, figs. 11 and 12. In one sample of sheep rumen contents two apparently diplococcoid organisms were seen to have reacted with the antiserum. Organisms which have reacted with this Gram-negative rod antiserum have not been found very often in the rumen contents tested.

DISCUSSION

The results given above were part of a preliminary survey of the possibilities of this method of identifying rumen organisms *in situ*. A selection of rumen organisms of differing morphology has been used and it has been possible to prepare conjugated antisera which can be shown visibly to have reacted with all these bacteria in pure culture. This reaction has shown the same, or serologically similar, organisms to be present in the gut contents of a number of animals. The method cannot be guaranteed to be entirely specific for one organism, but by suitable absorption of the antisera all gross non-specific fluorescence can be obviated and cross-reactions between different genera of bacteria seem to be rare. The reactions mentioned above were the only ones seen in the many slides examined. Some indeterminate masses of green fluorescent material are sometimes found in the rumen contents, but comparison with pure cultures kept under different conditions suggests that this fluorescent material is often disintegrating bacterial cells. Further work is now in progress to extend this method to different problems in rumen bacteriology.

The authors would like to thank Miss M. Garvock for assistance in preparing sera and Miss J. M. Eadie for allowing us to take some calf rumen samples.

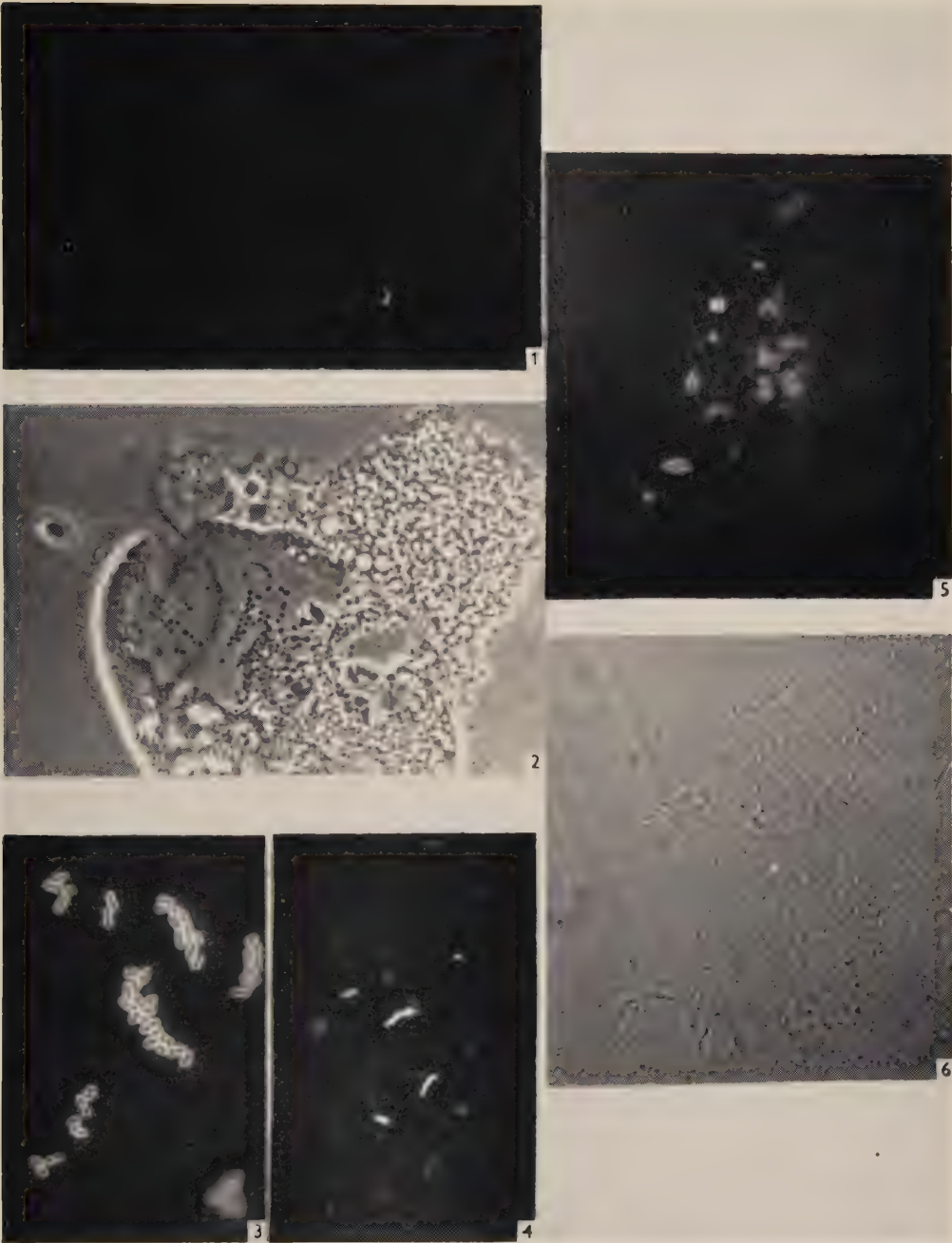
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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. A burst protozoan, etc., in rumen contents of a sheep treated with fluorescent normal rabbit serum. The materials showing up fluoresced white and bluish. Ultraviolet irradiation; $\times 680$.
- Fig. 2. The same field as in fig. 1 in white light. Phase contrast; $\times 680$.
- Fig. 3. Pure culture of a rumen strain of *Streptococcus bovis* reacting with fluorescent anti-serum. Ultraviolet irradiation; $\times 784$.
- Fig. 4. Mixed culture of *Lactobacillus* spp. reacting with fluorescent antiserum. Ultraviolet irradiation; $\times 680$.
- Fig. 5. Streptococci in rumen contents of a sheep reacting with fluorescent antiserum. Ultraviolet irradiation; $\times 784$.
- Fig. 6. The same field as fig. 5 in white light; $\times 784$.



P. N. HOBSON AND S. O. MANN—IDENTIFICATION OF RUMEN BACTERIA. PLATE 1
(Facing p. 470)

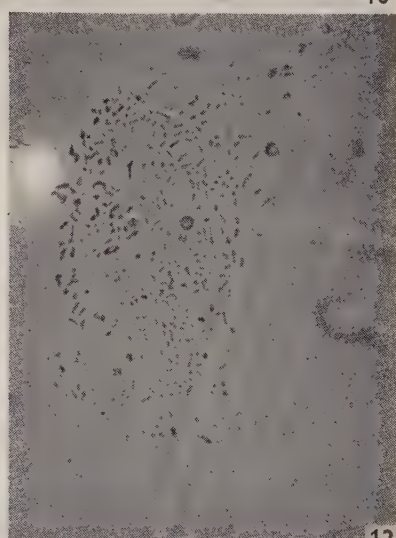
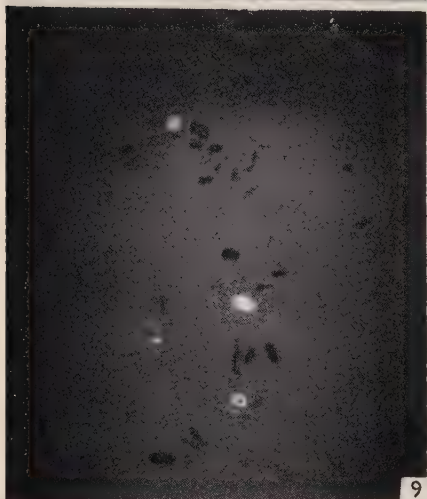
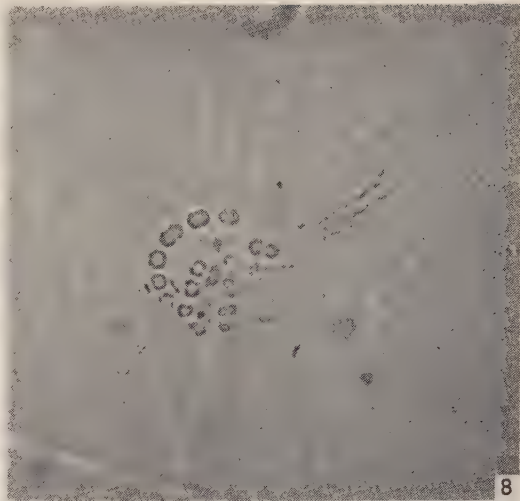
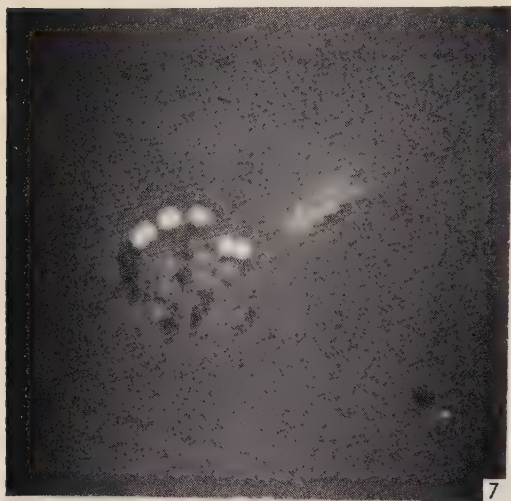


PLATE 2

- Fig. 7. Facultative anaerobic Gram-negative coccus (*Sarcina bakeri*) in rumen contents of a sheep reacting with fluorescent antiserum. The piece of fibre in the field fluoresced blue. Ultraviolet irradiation; $\times 980$.
- Fig. 8. The same field as fig. 7 in white light; $\times 980$.
- Fig. 9. Anaerobic Gram-negative coccus in the rumen contents of a calf reacting with fluorescent antiserum. Ultraviolet irradiation; $\times 850$.
- Fig. 10. The same field as in fig. 9 in white light. Some of the organisms in the field were motile and had moved between the two exposures. $\times 850$.
- Fig. 11. Gram-negative rods in the rumen of a sheep reacting with fluorescent antiserum. Ultraviolet irradiation; $\times 980$.
- Fig. 12. The same as fig. 11 in white light; $\times 980$.

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The Enzymic Conversion of the Tartaric Acids to Oxaloacetic Acid

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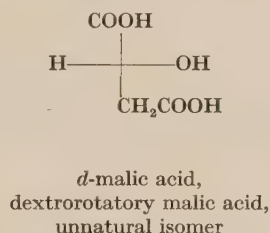
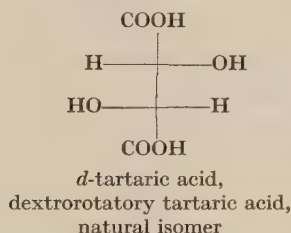
SUMMARY: Certain bacteria of the genus *Pseudomonas* attack the tartaric acids by means of inducible stereospecific dehydrases. Each dehydrase converts its specific isomeric substrate to oxaloacetic acid; in crude cell-free extracts the oxaloacetic acid is in turn converted to pyruvic acid, which accumulates. By treatment of the crude extracts with ethylenediaminetetraacetic acid (EDTA), substantial accumulations of oxaloacetic acid can be obtained from the *meso*- and *d*-tartaric acids, and assays for these two dehydrases, based on the accumulation of oxaloacetic acid in the presence of EDTA, have been developed. This procedure cannot be used to assay the *l*-tartaric acid dehydrase, which is itself very sensitive to EDTA. The patterns of inhibition of the three dehydrases by compounds sterically related to their substrates have been explored, and the findings are interpreted in terms of the minimal steric requirements for enzyme-substrate combination.

Many micro-organisms can attack the enantiomorphs of tartaric acid, usually with some degree of steric specificity (Pasteur, 1860, 1863; Barker, 1936; Vaughn, Marsh, Stadtman & Cantino, 1946; Stadtman, Vaughn & Marsh, 1945; Tabachnick & Vaughn, 1948; Mercer & Vaughn, 1951; Nomura & Sakaguchi, 1955). Surprisingly, there is only one brief report of microbial attack on *meso*-tartaric acid (Martin & Foster, 1955). Until recently, the biochemical mechanisms involved in the metabolism of the tartaric acids have been unknown. Barker (1936) proposed that the dissimilation of *d*-tartaric acid by *Aerobacter aerogenes* proceeds through oxaloacetate and pyruvate. On the basis of somewhat indirect evidence, the occurrence of this pathway in fluorescent pseudomonads was suggested by Nomura & Sakaguchi (1955) and by la Rivière (1956). While the work reported here was in progress, it was shown by Krampitz & Lynen (1956) that the dissimilation of *d*-tartaric acid by an unidentified Gram-positive rod involves a primary dehydration to oxaloacetic acid. A different pathway for the metabolism of the *l*- and *meso*-tartaric acids exists in animal mitochondria (Scholefield, 1955; Kun & Hernandez, 1956; Kun & Davies, 1956; Kun, 1956). Here, the primary attack occurs by dehydrogenation to oxaloglycollic acid.

This paper describes the enzymic mechanisms of tartaric acid metabolism by a series of *Pseudomonas* strains, which differ in the specificity of their attack on the three isomers. The existence of strains completely specific for a single isomer greatly facilitated the investigation of this problem. In an accompanying paper (Shilo & Stanier, 1957), certain physiological properties of the organisms concerned are described.

Configurational notation

In the chemical literature, the configurations assigned to the enantiomorphs of tartaric acid are inconsistent: some authors designate the (+)-isomer as L-(+)-tartaric acid, others as D-(+)-tartaric acid. As Buchanan (1951) pointed out, this ambiguity is an inevitable consequence of the current configurational notation. From the reference standard, D-(+)-glyceraldehyde, one can derive (-)-tartaric acid, via D-(-)-threose; but one can also derive (+)-tartaric acid, via D-(-)-glyceric acid, (+)-isoserine and (+)-malic acid. This confused situation can only be remedied by the adoption of a new series of conventions for the specification of configuration, such as those proposed by Cahn & Ingold (1951). In the meantime, the biologist is faced with a dilemma. We shall resolve it by referring to the tartaric acids and the related substances discussed in this and the ensuing paper in terms of their rotations, as *d*-tartaric acid, *d*-malic acid, etc. The configurational relationships, using the Fischer convention, are indicated by the following structures:



METHODS

Organisms and conditions of cultivation. *Pseudomonas* strains *meso* 1, *d* 15 and *l* 1 were used. They were isolated by elective culture with *meso*-, *d*- and *l*-tartaric acids, respectively. In all cases, the attack on the isomers of tartaric acid was strictly and specifically inducible. Strains *meso* 1 and *d* 15 can attack only *meso*- and *d*-tartaric acid, respectively. Strain *l* 1 can be induced to attack either *d*- or *l*-tartaric acid, by growth in the presence of the appropriate isomer.

The basal medium used for the preparation of specifically induced organisms consisted of: Na tartrate, 5.0 g.; NH₄Cl, 1 g.; MgSO₄·7H₂O, 0.05 g.; CaCl₂·2H₂O, 0.05 g.; FeCl₃, 0.05 g.; Difco yeast extract, 0.025 g.; phosphate buffer (0.01 M, pH 7.2), 1000 ml. Cultures were incubated at 30° on a mechanical agitator in Fernbach flasks (2 l. capacity) containing 1000 ml. medium. Organisms were harvested before termination of the exponential phase of growth.

Chemicals. *d*-Malic acid (the unnatural isomer) and *d*-, *l*- and *meso*-isomers of tartaric acid were purchased from the California Foundation for Biochemical Research. The *cis* and *trans* isomers of *dl*-epoxysuccinic acid were kindly furnished by Dr J. W. Foster.

Preparation of extracts. The organisms from 1 l. of culture medium were harvested by centrifugation, washed once with phosphate buffer (pH 7.2,

0.02 M), and resuspended at a density of 4–5% (w/v) in the same buffer. This suspension was then disintegrated by treatment for 10 min. in an ice-cooled Raytheon 9-KC sonic oscillator. Coarse debris and the particulate fraction were sedimented by centrifugation for 30 min. at 19,000 g, yielding a clear straw-coloured supernatant liquid which contained *c.* 10 mg. protein/ml. Such extracts were used in all the experiments to be described.

Chemical methods. Pyruvic acid and total keto acids were estimated colorimetrically by the method of Friedman & Haugen (1943), and chromatographically by the method of Cavallini, Frontali & Toshi (1949). β -Ketoacids were measured manometrically by catalytic decarboxylation with 4-aminoantipyrine (Akamatsu, 1950; Sistrom & Stanier, 1953). Oxaloacetic was determined quantitatively by light absorption at 280 m μ . with a Beckman model DU spectrophotometer (Green, Leloir & Nocito, 1945; Cammarata & Cohen, 1951). *d*- and *l*-Tartaric acids were estimated colorimetrically by the metavanadate method (Matchett, Legault, Nimmo & Notter, 1944), and detected chromatographically on Whatman no. 1 paper after development with butanol/water/acetic acid mixture (50:40:10) by spraying with bromophenol blue. Protein estimations were made by Pardee's modification of the biuret method (Pardee, personal communication). Manometric measurements of oxygen uptake and CO₂ evolution were carried out in the conventional Warburg apparatus at 30° with air as the gas phase.

RESULTS

The formation of oxaloacetic acid and pyruvic acid from the tartaric acids

Crude extracts, centrifuged at gravitational fields insufficient to sediment all the cytochrome-bearing particles, can oxidize specifically the tartaric acid isomers for which the bacteria were induced; however, the very high endogenous respiration makes the data difficult to interpret. When extracts were thoroughly clarified by centrifugation, they showed a very low endogenous oxygen consumption, which was only slightly increased by the addition of tartaric acid. However, with such extracts a rapid evolution of CO₂ resulted from the addition of the appropriate isomer of tartaric acid; the total amount of CO₂ evolved amounted to slightly more than one mole/mole tartaric acid supplied.

Typical manometric data, obtained with an extract from strain *meso* 1 acting upon *meso*-tartaric acid, are shown in Fig. 1. The same type of manometric data was obtained with extracts of strains *d* 15 and *l* 1, acting upon the *d*- and *l*-isomers, respectively. Chromatographic analysis showed that the tartaric acid furnished had disappeared completely from the reaction mixture at the time when the evolution of CO₂ had ceased. These observations establish that the primary attack on all three isomers is a non-oxidative one. In each case, chemical analysis of the reaction mixture showed that pyruvic acid had accumulated in appreciable quantities; it was the only major keto-acid present (Table 1).

The formation of CO₂ and pyruvic acid from tartaric acid suggested that the

first product formed might be oxaloacetic acid, which did not accumulate because of its rapid decarboxylation. Accordingly, the effect of ethylenediamine tetraacetic acid (EDTA) on tartrate breakdown was tested. Since EDTA chelates Mg^{++} , it might be expected to inhibit the decarboxylation of oxaloacetic acid.

Table 1. *The formation of pyruvic acid from the three isomers of tartaric acid in the presence of specific bacterial extracts*

The incubation mixtures contained 100 μ mole sodium tartrate and 0.5 ml. of enzyme extract in phosphate buffer (0.02 M, pH 7.2) in a total volume of 2 ml. The mixtures were incubated at 30° for 10 min., after which the reactions were stopped by adding 2 ml. of 10 % trichloroacetic acid. Pyruvate was estimated in the deproteinized supernatant fluid by the Friedman & Haugen (1943) method, with xylene for extraction of the ketoacid.

Substrate	Source of extract (strain)	Pyruvate found (μ mole)	E420/E520
<i>d</i> -Tartaric acid	<i>d</i> 15	12.7	1.37
<i>l</i> -Tartaric acid	<i>l</i> 1	11.6	1.38
<i>meso</i> -Tartaric acid	<i>meso</i> 1	26.7	1.31

The addition of EDTA (final concentration 0.04 M) to an extract from strain *meso* 1 virtually abolished the evolution of CO_2 from *meso*-tartaric acid (Fig. 2). This was accompanied by the accumulation of large quantities of β -ketoacid, as shown by the large amount of CO_2 evolved upon addition of 4-aminoantipyrine to the reaction mixture after 15 min. of incubation; in the absence of EDTA, the accumulation of β -ketoacid was negligible (Fig. 2). The 2,4-dinitrophenyl hydrazones of the ketoacids accumulating from *meso*-tartrate in the presence and absence of EDTA were prepared and subjected to chromatographic analysis. The reaction mixture without EDTA yielded only the 2,4-dinitrophenylhydrazone of pyruvic acid; the reaction mixture with EDTA yielded also a second 2,4-dinitrophenylhydrazone, chromatographically indistinguishable from the 2,4-dinitrophenylhydrazone of oxaloacetic acid. Furthermore, analysis by the method of Friedemann & Haugen showed that in the extract treated with EDTA, the total quantity of ketoacid was almost double the quantity of pyruvic acid (Table 2).

Table 2. *The accumulation of ketoacids from meso-tartaric acid in the presence and absence of EDTA*

The incubation mixtures contained: 20 μ mole *meso*-tartaric acid; 0.5 ml. of *meso* 1 extract; and phosphate buffer (0.02 M, pH 7.2) to make 1.5 ml. The reactions were stopped by the addition of 1.5 ml. of 10 % trichloroacetic acid after 15 min. of incubation at 30°. Pyruvate was estimated in the deproteinized supernatant by the method of Friedman & Haugen (1943) in xylene extracts. For total ketoacid estimation, ethyl acetate was used as solvent.

Treatment	Pyruvate (μ mole)	Total ketoacids (μ mole)
No EDTA added	18.0	17.4
10 μ mole EDTA	6.9	11.05

By similar methods, the extract from strain *d* 15 was shown to produce oxaloacetic acid from *d*-tartaric acid in the presence of 0.04 M EDTA. The extract from strain *l* 1, however, did not decompose tartaric acid in the presence of 0.04 M-EDTA. With lower concentrations of EDTA (0.004 M), the reaction proceeded and a small accumulation of oxaloacetic acid was demonstrated.

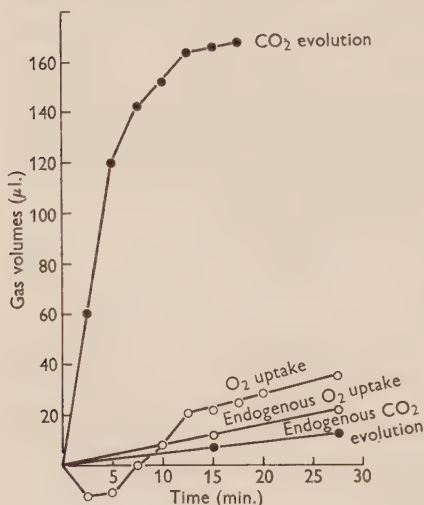


Fig. 1

Fig. 1. O₂ uptake and CO₂ evolution from *meso*-tartaric acid by an extract of strain *meso* 1. Incubation mixture contained: 5 μmole neutralized *meso*-tartrate; 0.5 ml. *meso* 1 extract; phosphate buffer (0.02 M, pH 7.2) to make 2 ml. Temperature 30°.

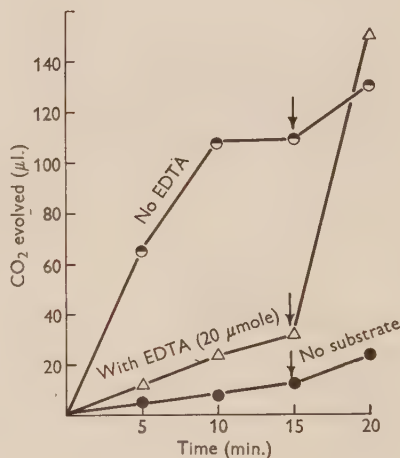


Fig. 2

Fig. 2. Effect of EDTA on CO₂ evolution and β-ketoacid accumulation from *meso*-tartaric acid by an extract from strain *meso* 1. Incubation mixtures contained: 5 μmole *meso*-tartaric acid; 0.5 ml. *meso* 1 extract; phosphate buffer (0.02 M, pH 7.2) to make 1.8 ml. Temperature 30°. At points indicated by arrows, the incubation mixture was acidified with 0.1 ml. N-acetic acid and 0.4 ml. 4-aminoantipyrine (0.1 M) was added.

It is accordingly evident that the decomposition of the three isomers of tartaric acid involves in each case a specific dehydration to oxaloacetic acid (probably the enol form), followed by decarboxylation of the oxaloacetic acid to pyruvic acid. Thus only the initial step in the metabolism of the tartaric acids is biochemically specific and unique; with the formation of oxaloacetic acid and pyruvic acid, the main pathways of intermediary metabolism are attained. We shall term the specific enzymes involved tartaric acid dehydrases.

The development of assays for the tartaric acid dehydrases

Oxaloacetic acid can be estimated spectrophotometrically by its light absorption at 280 mμ. Since it accumulates when *meso*-tartaric acid is enzymically attacked in the presence of EDTA, a spectrophotometric assay which measures the accumulation of oxaloacetic acid directly was developed. Fig. 3 shows the accumulation of oxaloacetic acid in the presence and in the absence of EDTA (10 μmole) as estimated from optical density changes at 280 mμ.

In the absence of EDTA, there was an initial rapid formation of oxaloacetic acid from *meso*-tartaric acid, followed by a decline. The maximal quantity of oxaloacetic acid accumulated from 5 μ mole *meso*-tartrate was less than 2 μ mole. In the presence of EDTA, oxaloacetic acid accumulated steadily, more than 4 μ mole being formed from 5 μ mole *meso*-tartrate, and more than 8 μ mole from 10 μ mole *meso*-tartrate. At the points indicated by arrows in

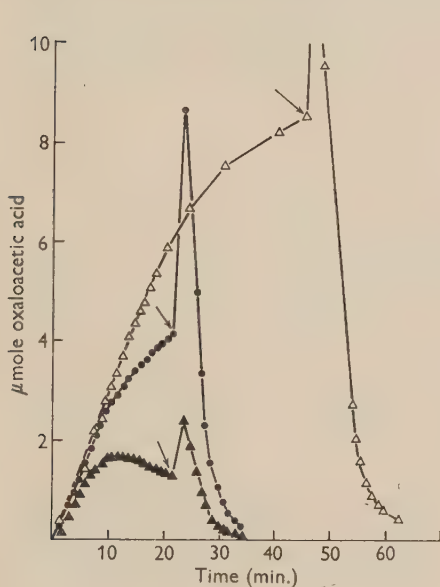


Fig. 3

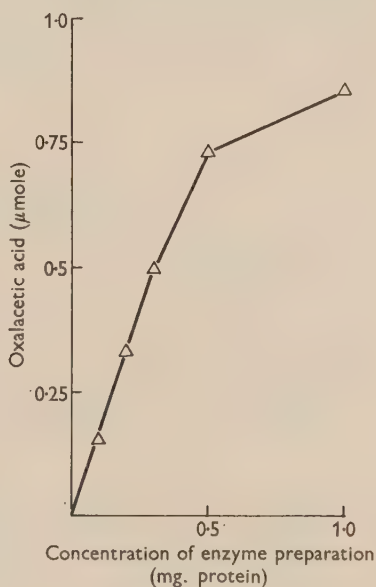


Fig. 4

Fig. 3. Effect of EDTA on the accumulation of oxaloacetic acid from *meso*-tartaric acid by an extract from strain *meso* 1. An incubation mixture contained 0.05 ml. *meso* 1 extract and phosphate buffer (0.02 M, pH 7.2) to make 2.5 ml., ▲—▲, 5 μ mole *meso*-tartrate; ●—●, 5 μ mole *meso*-tartrate + 10 μ mole EDTA; △—△, 10 μ mole *meso*-tartrate + 10 μ mole EDTA. The accumulation of oxaloacetic acid was followed at 280 m μ . in the Beckman spectrophotometer. Mg⁺⁺ (10 μ mole) was added at points indicated by arrows.

Fig. 4. The effect of enzyme concentration on the rate of accumulation of oxaloacetic acid from *meso*-tartaric acid. Incubation mixtures containing 10 μ mole of neutralized *meso*-tartaric acid, 10 μ mole EDTA, varying amounts of *meso* 1 extract and phosphate buffer (0.02 M, pH 7.2) were tested for differences in the rate of accumulation of oxaloacetic acid, as measured in the Beckman spectrophotometer at 280 m μ .

Fig. 3, 10 μ mole of MgCl₂ were added to the reaction mixtures. In each case there was an immediate rapid rise in optical density, a phenomenon which is also observed when Mg⁺⁺ is added to neutral aqueous solutions of pure oxaloacetic acid. The explanation for this is unknown, but it possibly reflects formation of a magnesium-complex with oxaloacetic acid. Following this initial increase in optical density, the optical density of the reaction mixture fell rapidly to a negligible value, reflecting the decarboxylation of oxaloacetic acid to pyruvic acid.

From Fig. 3, it can be seen that with a given quantity of enzyme, the initial rate of accumulation of oxaloacetic acid in the presence of EDTA was virtually identical for 5 and 10 μ mole *meso*-tartrate. The effect of enzyme concentration on the initial rate of formation of oxaloacetic acid in the presence of EDTA is shown in Fig. 4; within limits, there is a linear relationship between enzyme concentration and activity. Hence it is evident that the spectrophotometric method provides a convenient continuous assay procedure for the *meso*-tartaric acid dehydrase in the presence of EDTA. With *d*-tartaric acid dehydrase, the same assay method can be used, although the accumulation of oxaloacetic acid is never so complete, and the rate of its formation starts to

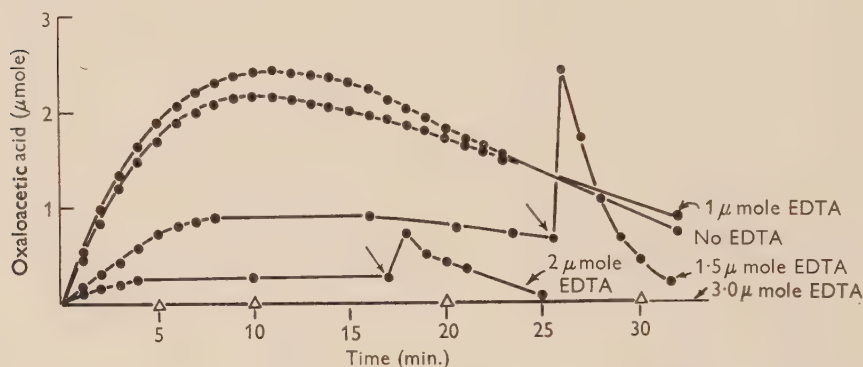


Fig. 5. Effect of EDTA on *l*-tartrate dehydrase. Incubation mixtures contained: 0.04 ml. extract of strain *l* 1 grown on *l*-tartrate medium; 10 μ mole *l*-tartrate; different amounts (0 to 3 μ mole) of EDTA; phosphate buffer (0.02 M, pH 7.2) to make 2.5 ml. At times indicated by arrows, 10 μ mole of Mg^{++} were added. Accumulation of oxaloacetic acid was followed in the Beckman spectrophotometer at 280 m μ .

decline at an earlier point in the reaction. Although the accumulation of oxaloacetic acid from *l*-tartaric acid in the presence of the specific dehydrase can be demonstrated, this dehydrase is itself strongly inhibited by EDTA (Fig. 5). The assay of this enzyme can be made, although somewhat imprecisely, by measuring the initial rate of accumulation of oxaloacetic acid in a reaction system without EDTA.

General properties of the tartaric acid dehydrases

The stereospecificity of the three tartaric acid dehydrases is absolute: each enzyme is capable of attacking only one isomer of tartaric acid. Furthermore, no other substrates for any of these enzymes have been found. The reactions catalysed by the *meso*- and *d*-tartaric acid dehydrases appear to be essentially irreversible: when the enzymes preparations were incubated with oxaloacetic acid, the decrease in optical density at 280 m μ . was not greater than that observed in controls containing oxaloacetic acid alone. The reversibility of the reaction catalysed by the *l*-tartaric acid dehydrase cannot be tested in this way, since the decarboxylation of oxaloacetic acid cannot be blocked by EDTA treatment.

Experiments with the dehydrases were usually conducted at pH 7.2 in phosphate buffer. The effect of pH value on the activity of the *meso*-tartaric acid dehydrase was measured; there was a broad plateau between pH 6.9 and 8.5, with a rapid decline of activity on both sides.

Attempts to purify the *meso* dehydrase have so far failed. Dialysis and ammonium sulphate precipitation both caused great loss of activity, somewhat decreased by the presence of EDTA. Nevertheless, in crude extracts the *meso* dehydrase is stable for several weeks when kept frozen. The *d*- and *l*-tartaric acid dehydrases, on the other hand, lost considerable activity when stored for a few days in the frozen state.

Table 3. *Patterns of inhibition of the tartaric acid dehydrases by sterically related compounds*

The incubation mixtures contained: 10 μ mole of specific substrate, 10 μ mole of the listed compound, and phosphate buffer (0.02 M, pH 7.2) to make 2.5 ml. In the cases of the *meso* dehydrase and the *d* dehydrase, 10 μ mole of EDTA were also added. The rate of accumulation of oxaloacetic acid was estimated at 280 m μ . by the Beckman spectrophotometer, in the presence and absence of the compounds tested as inhibitors.

Inhibitor	% inhibition at an equimolar ratio to substrate		
	<i>Meso</i> dehydrase	<i>d</i> dehydrase	<i>l</i> dehydrase
<i>meso</i> -Tartaric acid	—	54	93
<i>d</i> -Tartaric acid	74	—	35
<i>l</i> -Tartaric acid	0	0	—
Citraconic acid	0	—	—
<i>d, l</i> -Citromalic acid	0	—	—
Itaconic acid	0	—	—
<i>d, l-trans</i> -Epoxy succinic acid	16	56	88
<i>d, l-cis</i> -Epoxy succinic acid	87	0	16
Tartronic acid	42	66	—
<i>d</i> -Malic acid	74	72	—
<i>l</i> -Malic acid	0	0	—

Inhibition pattern. In addition to their stereospecificity with respect to substrate, the three tartaric acid dehydrases showed characteristic individual patterns of inhibition by compounds sterically related to the substrates (see Table 3). The data were obtained by determining the decrease in the rate of oxaloacetic acid accumulation which occurred when each of the listed inhibitors was added to the enzyme preparation in the presence of an equimolar quantity of the specific substrate. It can be seen that in some cases the degree of inhibition was exceedingly severe: for example, the *meso* dehydrase was inhibited to the extent of at least 74% by *d*-tartaric, *d*-malic and *d, l-cis*-epoxy-succinic acids, and the *l*-tartaric acid dehydrase was inhibited to an extent of 85% or more by *meso*-tartaric acid and *d, l-trans*-epoxy succinic acid. It seems likely that these inhibitors act competitively, although this has been definitely shown only for the inhibition of the *meso*-tartaric acid dehydrase by *d*-tartaric and tartronic acids.

DISCUSSION

The enzymic dehydration of tartaric acid to oxaloacetic acid, first established by Krampitz & Lynen (1956) for the *d*-isomer, occurs also with the *meso*- and *l*-isomers, and the attack on all three tartaric acids by bacteria of the genus *Pseudomonas* appears to occur principally by means of stereospecific dehydrases. In the present enzymic study, the existence of such stereospecific dehydrases was established for three strains of *Pseudomonas*. In addition, however, we have examined extracts from many other *Pseudomonas* strains which can attack one, two, or all three of the tartaric acids; in most cases oxaloacetic acid is an intermediate. This preliminary survey has shown, however, that at least one additional pathway for the metabolism of *d*-tartaric acid exists in pseudomonads. Extracts from certain *d*-specific strains did not yield oxaloacetic acid from *d*-tartaric acid in the presence of EDTA; instead, small amounts of a ketoacid similar in its properties to glyoxylic acid accumulated.

The patterns of inhibition observed with the three tartaric acid dehydrases can be interpreted, at least in part, in steric terms. We shall consider first the inhibitory interactions between the three tartaric acids. *meso*-Tartaric acid, the internally compensated molecular form, can be regarded as containing both the *d* and the *l* configurations. The fact that the *meso* dehydrase is strongly inhibited by the *d*-isomer of tartaric acid, but not at all by the *l*-isomer, suggests that it is that portion of the *meso*-tartaric acid molecule that contains the *d*-configuration which is actually bound to the *meso* dehydrase. The *d* dehydrase is inhibited by the *meso*-isomer but not by the *l*-isomer, while the *l* dehydrase is inhibited by the *meso*-isomer but not by the *d*-isomer. These facts suggest that the combining sites of the tartrate dehydrases are specific for the enantiomorphic configuration shown by their respective substrates; and, further, that only one of the asymmetric carbons of the substrate is involved in the enzyme-substrate combination.

These interpretations of the minimal steric configurations necessary for binding by the three dehydrases also fit the observed inhibitions with other substances sterically related to the tartaric acids. Thus *d*-malic acid inhibits the *meso* dehydrase and the *d* dehydrase. Tartronic acid, which has the essential structure of the postulated combining region of the substrates, but lacks asymmetry, also inhibits the *meso* and *d* dehydrases. Lastly, there are the *trans*- and *cis*-epoxysuccinic acids to be considered. The *dl*-*cis* form strongly inhibits only the *meso* dehydrase, while the *dl*-*trans* form strongly inhibits the *d* and *l* dehydrases, but only slightly the *meso* dehydrase. Here it seems evident that geometrical configurations also play a role in the inhibitory process; until the enantiomorphs of each geometrical isomer have been tested separately, a detailed analysis in steric terms cannot be made.

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The Utilization of the Tartaric Acids by Pseudomonads

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SUMMARY: *Pseudomonas* strains, capable of utilizing as carbon sources one or more isomers of tartaric acid, were isolated from soil by specific elective culture methods. Most strains were markedly stereospecific, utilizing only the isomer or isomers upon which they had developed during the elective cultivation. Enzymic study showed that the commonest mechanism for the dissimilation of the tartaric acids by this group of strains is dehydration to oxaloacetic acid, through the action of stereospecific dehydrases. The attack on the tartaric acids is strictly inducible, and in many strains the induced state is rapidly lost in the absence of the substrate-inducer. Enzymic assay showed that such loss of induction cannot be accounted for by loss of the specific dehydrase, which is often present in large quantities in organisms which have become incapable of attacking the substrate. This indicates that the metabolism of the tartaric acids requires specific inducible transport systems for bringing the tartaric acids into the cell, in addition to the specific, inducible dehydrases. The existence of such transport systems is further indicated by the fact that the inhibitory interactions between the isomers of tartaric acid, demonstrable with the cell-free dehydrase preparation, do not exist *in vivo*.

Although there has been much work on the utilization of tartaric acids by other groups of micro-organisms, information about the metabolism of these acids by pseudomonads is very limited. Nomura (1953*a-c*) and Nomura & Sakaguchi (1955) reported that a strain of *Pseudomonas incognita* attacked *d*-tartaric acid* under both aerobic and anaerobic conditions and la Rivière (1956) reported similar findings with another *Pseudomonas* sp. The isolation of pseudomonads capable of oxidizing the three isomers of tartaric acid has been undertaken and the results are described here.

METHODS

Unless otherwise specified, the methods employed were those described by Shilo (1957).

Elective cultivation. The elective media used consisted of: NH_4Cl , 1.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g.; FeCl_3 , 0.05 g.; Yeast extract (Difco), 0.05 g.; phosphate buffer (M/150, pH 7.2), 1 l. This basal medium was supplemented with 1 g. of an isomer of tartaric acid. Shallow layers of liquid medium in Erlenmeyer flasks were inoculated with soil and incubated at 30° without agitation. After 24-48 hr., transfers were made to second flasks containing media of the same composition. After growth had occurred in the second flasks, streaks were made on plates of the same medium solidified with agar. Experience showed that the organisms isolated by enrichment with one isomer of tartaric acid were in general not able to attack the other isomers.

* For a discussion of the configurational notation employed, see Shilo (1957).

In order to obtain strains capable of attacking two or three isomers of tartaric acid, the elective procedure was slightly modified. After an elective culture had been established with one isomer, a transfer was made to a flask of similar medium with a second isomer, and this, in turn, was transferred after growth to a flask of elective medium with the third isomer. By such successive elective cultures, it was easy to obtain strains which attacked two or three isomers of tartaric acid.

RESULTS

Table 1 shows the patterns of utilization of the tartaric acids and related acids as carbon and energy sources for growth by the pseudomonads which were isolated. Where the pathway of utilization of tartaric acid has been established through enzymic study, the nature of this pathway is indicated. It can be seen that attack on the tartaric acids by dehydration to oxaloacetic acid is the most common pathway in this group, although there are two strains for which a different pathway is indicated from preliminary enzymic observations. A very striking feature of the patterns of utilization shown in Table 1 is the specificity of many strains with respect to utilization of the three tartaric acids. Two sets of elective cultures with either *d*- or *meso*-tartrate yielded only strains capable of attacking the isomer used for election; in one elective culture with the *l*-isomer, strains capable of growth on both enantiomorphs were found.

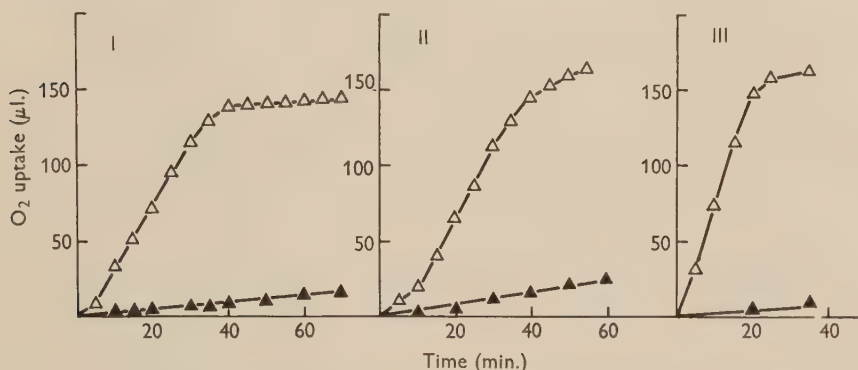


Fig. 1. The specificity of oxidation of the tartaric acids by three pseudomonad strains. Oxygen uptakes by suspensions furnished with 5 μ mole substrate. (I) Strain *l* 1, grown on *l*-tartrate. \triangle — \triangle , *l*-tartrate; \blacktriangle — \blacktriangle , endogenous, *d*-tartrate, *meso*-tartrate. (II) Strain *d* 15, grown on *d*-tartrate. \triangle — \triangle , *d*-tartrate; \blacktriangle — \blacktriangle , endogenous, *l*-tartrate, *meso*-tartrate. (III) Strain *meso* 1, grown on *meso*-tartrate. \triangle — \triangle , *meso*-tartrate; \blacktriangle — \blacktriangle , endogenous, *l*-tartrate, *d*-tartrate.

Pattern of induction

In every case, the attack on the tartaric acids was a strictly inducible one: the tartrate-utilizing strains were incapable of attacking tartaric acid immediately after growth on succinate or acetate. Fig. 1 shows the specificity of attack on the tartaric acids by strains which were isolated through elective culture with *meso*-, *l*- and *d*-tartrate, respectively. In this experiment, each strain had been grown at the expense of the isomer of tartaric acid used for its election.

Table 1. *Growth patterns of the different strains of Pseudomonas obtained from elective cultures with the tartaric acids*

Growth on the following substrates as sole carbon and energy sources*

Strains†	<i>meso-</i>					Citra- conic acid	Itaconic acid	<i>d</i> -Malic acid	Fluores- cent pigment produc- tion‡	Pathway of tartrate breakdown
	<i>d</i> -Tar- taric acid	<i>l</i> -Tar- taric acid	Tar- taric acid	Maleic acid	Fumaric acid	Acetic acid	Mesa- conic acid			
<i>d</i> 15	+	—	—	—	+	+	+	—	—	Oxaloacetate (OA) pathway
<i>d</i> 16	+	—	—	—	+	+	—	—	+	Glyoxylate pathway
<i>l</i> 1	+	+	—	—	+	+	—	—	+	OA pathway for <i>d</i> - and <i>l</i> -
<i>l</i> 2	+	+	—	—	+	+	+	—	+	OA pathway for <i>d</i> - and <i>l</i> -
<i>meso</i> 1	—	—	+	—	+	+	—	—	+	OA pathway for <i>d</i> - and <i>l</i> -
<i>meso</i> 2	—	—	+	—	+	+	—	—	—	—
<i>meso</i> 3	—	—	+	—	+	+	—	—	—	—
<i>mmd</i>	+	+	+	—	+	+	—	—	+	OA pathway for all three isomers
<i>mmal</i>	+	+	+	—	+	+	—	—	+	OA pathway for all three isomers
<i>dmm</i>	+	+	+	—	+	+	—	—	—	—
Maleic 5	+	—	—	+	+	+	—	—	—	Glyoxylate pathway
Fumaric 4	—	—	±	—	+	+	+	—	—	—
<i>d</i> -Malic 1	+	—	—	—	+	+	—	—	+	OA pathway
<i>lml</i>	+	+	+	—	+	+	—	—	—	—
<i>dmd</i>	+	—	+	—	+	+	—	+	—	—

* Growth was tested on mineral base agar containing 0.1 % of the specified substrate; incubation at 30° for 24–48 hr.

† The prefixes of the strain designations indicate the modes of election used. For example, *d* strains were isolated by simple election with *d*-tartaric acid, fumaric strains by simple election with fumaric acid. Strains such as *mmd*, *dmm*, and *lml* were obtained by successive election with more than one isomer of tartaric acid. Strain *mmd*, for example, was isolated after successive transfers in elective cultures with *meso*-tartaric acid, *meso*-tartaric acid, and *d*-tartaric acid.

‡ Fluorescent pigment production was examined on yeast extract (0.5 %) slopes.

It will be noted that strain *l* 1, although capable of growth at the expense of either enantiomorph, did not immediately attack the *d*-isomer after growth on *l*-tartaric acid. When this strain was tested for the utilization of the enantiomorphs after growth on *d*-tartaric acid, there was an immediate rapid attack on the *d*-isomer and a very weak, but significant, attack on the *l*-isomer. In all cases, the total oxygen uptake/mole tartrate oxidized was slightly more than 1 mole, i.e. about 40% of the theoretical value for complete oxidation. This probably reflected the occurrence of oxidative assimilation.

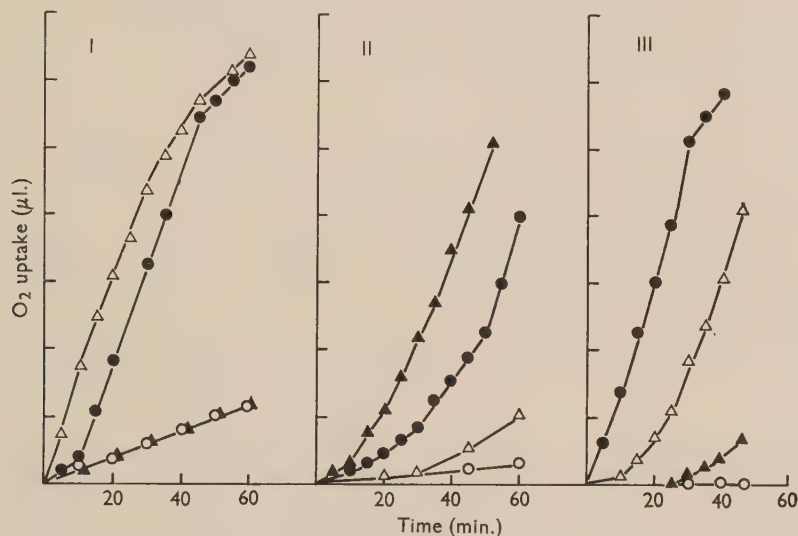


Fig. 2. Patterns of induction for oxidation of the tartaric acids by a pseudomonad strain (*mmdl*) capable of utilizing all three isomers. (I), grown on *l*-tartrate; (II), grown on *d*-tartrate; (III), grown on *meso*-tartrate. $\circ-\circ$, endogenous; $\blacktriangle-\blacktriangle$, 10 μ mole *d*-tartrate; $\triangle-\triangle$, 10 μ mole *l*-tartrate; $\bullet-\bullet$, 10 μ mole *meso*-tartrate.

In Fig. 2, the pattern of oxidation of the tartaric acids by a strain capable of growing on all three isomers is shown. The specificity of induction by the isomer used for growth is evident. There is one curious feature of the induction patterns: growth on *meso*-tartaric acid appears greatly to accelerate induction for the *l*-isomer, and vice versa. This effect cannot be explained by non-specific induction; extracts of cells grown on the *meso*-isomer do not contain *l*-tartaric acid dehydrase, and extracts of the cells grown on the *l*-isomer do not contain *meso*-tartaric acid dehydrase.

The loss of induction

Early in the course of experiments on inductive patterns, it was observed that induction for the attack on *d*- and *meso*-tartrate was very readily lost. This phenomenon was most easily observed in cultures grown with limiting amounts of tartrate. Since tartrate was the sole source of carbon and energy, its depletion caused the population to enter the stationary phase, and this was invariably followed by a loss of the capacity for immediate attack on tartrate. The later the organisms are harvested in the stationary phase, the greater

becomes the lag before tartrate can be oxidized at a rapid rate. For example, organisms which have remained in the stationary phase for 4 hr. after tartrate depletion show, when harvested and tested manometrically, an absolute lag of 20 min. before tartrate is oxidized at all, and oxidation at maximal rate is established only after 50–60 min. (Fig. 3). It can easily be shown, by testing the dehydrase activity of extracts from these organisms, that the inability to attack tartrate is not a reflexion of the complete loss of the specific dehydrase. Organisms which have remained in the stationary phase for 4 hr. still contain 50 % of the specific dehydrase activity found in the population at the end of the logarithmic phase of growth (Table 2). On the basis of the enzymic analysis, we would expect a diminution in the rate of tartrate oxidation by whole organisms, but not a total loss. Since, however, the primary step in the

Table 2. *The behaviour of whole organisms of Pseudomonas strain m 1 with respect to the oxidation of meso-tartrate, and the meso-tartrate dehydrase activity shown by extracts prepared from them.*

Data on whole organisms taken from Fig. 3. Dehydrase assays were performed as described by Shilo (1957), and specific activities of extracts were computed on the basis of protein content.

Age of culture at time of harvest (hr.)	Whole cells			Extracts	
	Initial rate of meso- tartrate oxidation, μ l. O ₂ /hr.	Duration of lag before initiation of oxidation (min.)	Effect of an equimolar quantity of <i>d</i> -isomer on rate of meso- tartrate oxidation	Relative specific activity of meso-tartaric acid dehydrase	
				No <i>d</i> -isomer present	Equimolar quantity of <i>d</i> -isomer present
10.5	200	0	None	100	20
14.5	0	20	None	48	11
18.5	0	40	None	25	7

attack on tartrate is a non-oxidative one, it might be imagined that the total loss of the ability to consume oxygen at the expense of tartrate reflects the loss of the ability to attack the first oxidizable intermediate, even though tartrate itself can still be broken down. This explanation could be excluded by the demonstration that such organisms can immediately consume oxygen when furnished with oxaloacetate or pyruvate, the demonstrated metabolic intermediates in the dissimilation of tartrate. Clearly, therefore, the observed loss of induction cannot be explained on the basis of the loss of any of the known enzyme systems involved in the dissimilatory pathway for tartrate. It seems necessary to postulate the existence of an additional inducible system, required for transporting exogenous tartrate to the intracellular site of dehydrase action.

Discrepancies between inhibitory patterns shown in extracts and whole organisms

Further evidence for the assumption that the tartaric acids cannot freely penetrate from the environment to the site of dehydration without a preceding specific induction comes from the study of specific inhibition. As shown in the

accompanying paper by Shilo (1957), the *meso*-isomer of tartaric acid is a powerful inhibitor of the *d* dehydrase, and the *d*-isomer of the *meso* dehydrase. Yet when these inhibitors are tested with whole organisms, no inhibition, as measured in terms of oxygen uptake, can be shown. The absence of inhibition of the oxidation of *meso*-tartrate by the *d*-isomer *in vivo* is shown in Fig. 3, while Table 2 shows the marked inhibition (about 80 %) of the *meso* dehydrase by the *d*-isomer which occurs in extracts from these same organisms.

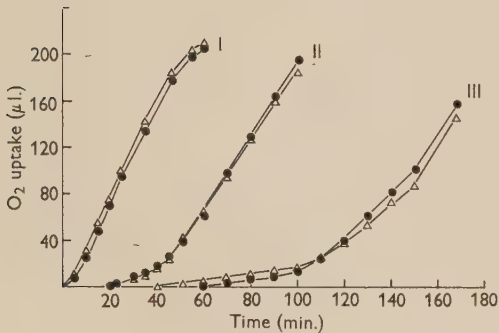


Fig. 3

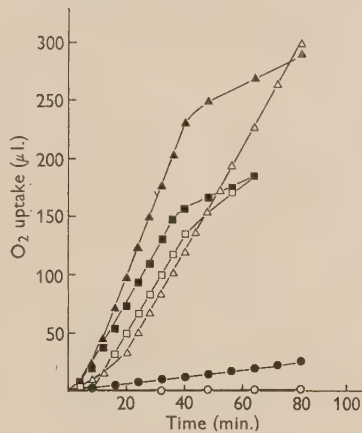


Fig. 4

Fig. 3. The oxidation of *meso*-tartrate by washed pseudomonads of strain *m* 1, harvested at various times from a medium containing *meso*-tartrate as sole carbon source. The culture entered the stationary phase at about 10.5 hr., because of *meso*-tartrate depletion. (I), organisms harvested at 10.5 hr.; (II) harvested at 14.5 hr.; (III) harvested at 18.5 hr. ●—●, oxygen uptake with *meso*-tartrate (10 μ mole); \triangle — \triangle , oxygen uptake with *meso*-tartrate (10 μ mole) + *d*-tartrate (10 μ mole). Endogenous respiration was negligible. Note the pronounced absolute lags in substrate oxidation after 14.5 hr. and 18.5 hr., and the absence of inhibition of *meso*-tartrate oxidation by *d*-tartrate.

Fig. 4. The inhibitory effect of *d*-tartrate on the oxidation of *meso*-tartrate by washed organisms of strain *mmdl* grown at the expense of *meso*-tartrate. ○—○, endogenous respiration; ■—■, *meso*-tartrate (5 μ mole); □—□, *l*-tartrate (5 μ mole); ▲—▲, *meso*-tartrate (5 μ mole) + *l*-tartrate (5 μ mole); ●—●, *meso*-tartrate (5 μ mole) + *d*-tartrate (5 μ mole); \triangle — \triangle , *d*-tartrate (5 μ mole) + *l*-tartrate (5 μ mole).

Inhibition of tartrate utilization by ultraviolet irradiation

The experiments reported above indicate that the tartaric acids cannot freely reach the intracellular sites of their breakdown by simple diffusion from the medium in which the organisms are suspended. However, the analysis of inductive patterns suggests that exposure to tartaric acid somehow changes the cells so that these substances can freely enter them. It is therefore tempting to assume that a special inductive event, the development of a specific carrier system, takes place when the cells are exposed to tartrate. In support of this assumption it has been observed that when organisms, harvested from a tartrate-grown culture in the stationary phase, are exposed to brief ultraviolet irradiation, they become almost completely incapable of regaining the ability

to oxidize tartrate. The dose of radiation employed had no effect on preformed enzyme systems, as shown by its inability to affect the oxidation of pyruvate. Since it is well known that ultraviolet irradiation has a highly selective inhibitory effect on enzyme synthesis, these findings suggest that a *de novo* act of enzyme formation is required in order for the cells to regain the ability to attack tartrate.

Inhibitory interactions in whole cells

In one strain (*mmdl*), it was possible to demonstrate the inhibition of the oxidation of *meso*-tartrate *in vivo*. The strain concerned can attack all three isomers of tartaric acid. When the organisms have been grown at the expense of *meso*-tartrate, the oxidation of this isomer can be almost totally inhibited by the simultaneous addition of an equimolar amount of the *d*-isomer (Fig. 4). However, the expected inhibition by the *meso*-isomer of the attack on the enantiomorphs, which is readily demonstrable with extracts of this strain, is not observed with whole organisms; the oxidations of *d*- and of *l*-tartaric acids by specifically induced organisms are unaffected by the presence of the *meso*-isomer. These observations can be explained by assuming that strain *mmdl* has a constitutive system for the transport of the *d*-isomer into the cell, but not for the transport of the *meso*-isomer. The dehydrases are all clearly inducible.

DISCUSSION

In the accompanying paper (Shilo, 1957) it was shown that the dissimilation of the tartaric acids by various pseudomonads involves the action of dehydrases, stereospecific with respect to substrate and inducer: both substrate and inducer-function for each dehydrase are shown by one, and only one, isomer of the tartaric acids. The experiments with whole organisms reported in this paper reveal, however, that the acquisition of the ability to attack any one of the isomers of tartaric acid is not solely a reflexion of the induced synthesis of the relevant dehydrase; dehydrase synthesis is a necessary, but not sufficient, event. It is evident that each isomer of tartaric acid also induces specifically the formation of a system which is required for the transport of the isomer in question from the external environment to the site of dehydrase action within the cell.

The specific induction of transport systems, required to bring substances to the site of enzyme action in bacteria, has been clearly established by a number of earlier investigators. Barrett & Kallio (1953) showed that the 'adaptive lag' in the utilization of exogenous citrate by *Pseudomonas fluorescens* cannot be explained by the lack of the relevant enzymes of the tricarboxylic acid cycle, which are present in extracts from cells 'unadapted' to oxidize citrate. Nor is the lag a reflexion of the existence of a mechanical permeability barrier, since Barrett, Larsen & Kallio (1953) found that inhibitors of enzyme synthesis (ultraviolet irradiation, amino acid analogues) could totally block the induction of ability to oxidize citrate. The fact that inhibition by amino acid analogues can be prevented by simultaneous addition to the system

of the relevant amino acid speaks particularly strongly for the necessity of a specific act of enzyme synthesis to make externally supplied citrate available to the intact organism. A more detailed study of the process of citrate utilization in *Aerobacter aerogenes* (Davis, 1956) revealed a similar situation. In *A. aerogenes*, it has been directly demonstrated that the intracellular accumulation of citrate occurs only after specific induction. Indeed, it seems very probable that much of the evidence obtained with whole organisms, which has tended to suggest that the tricarboxylic acid cycle is not functional in aerobic micro-organisms, can be best interpreted as reflecting the existence of barriers, only overcome by specific induction, which bar the entry into the cell of exogenous tricarboxylic acid cycle components.

Specific transport mechanisms in micro-organisms for other exogenous nitrilites have been shown. Thus, in *Escherichia coli* an inducible transport system clearly controls the entry of β -galactosides into the cell, and this system has provided the material for the most detailed and elegant experiments so far done on the general phenomenon (Monod, 1956; Dr Melvin Cohn, personal communication). Specific constitutive transport systems for the accumulation of various amino acids in bacteria have been shown (Cohen & Rickenberg, 1955). Indeed, the now rapidly accumulating evidence suggest that most of the nitrilites which the bacteria absorb from their environment must gain entry to the cell by a process of active transport, rather than of simple diffusion. The inducible control exerted over so many of these transport mechanisms is clearly of very great adaptive significance, since it confers on the microbial cell a high degree of plasticity and selectivity in the assimilation of compounds from its ever-changing external environment.

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Interference between Active and Ultraviolet-irradiated Rift Valley Fever Virus

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SUMMARY: Interference was demonstrated with pantropic Rift Valley fever virus in appropriately ultraviolet-irradiated mouse serum in which a residuum of active virus remained. The degree of interference varied with the amount of irradiation to which the virus had been exposed. With the smallest doses of ultraviolet radiation, which left a fair amount of active virus, the only manifestation of interference was a prolongation of the incubation period. With virus which had received optimal irradiation, concentrated inocula elicited no symptoms but the same material in higher dilution produces the typical fatal illness. With doses of ultraviolet radiation between the minimum and the optimum, virus suspensions were obtained which showed interference, but many of the mice developed signs of neurological involvement after a prolonged incubation period. Although the symptoms elicited were neurological in origin, the virus recovered from the brain was unaltered pantropic variety. The distribution of this virus in the affected mice was determined. It is suggested that the residual live virus contained in irradiated material undergoes a growth cycle in a limited number of liver and other susceptible cells not protected by the interference effect of inactivated virus. The late neurological manifestations are attributed to this newly formed virus and the lesser affinity of the brain cells for the interfering inactivated virus. Many of the mice which survived inoculations of concentrated irradiated virus were shown to be immune to subsequent infection with 100 MLD of Rift Valley fever virus. This immunity is considered to be due to a latent infection initiated by residual live virus present in the irradiated material. Virus subjected to prolonged irradiation probably loses its immunizing power because of complete inactivation rather than destruction of the antigenicity of individual virus particles.

When virus suspensions are inactivated by means of a relatively mild procedure such as appropriate doses of ultraviolet radiation, infective virus may still be present but on injection into susceptible animals may remain masked because of interference by inactivated virus. Examples of such 'auto-interference' were described by Ziegler, Lavin & Horsfall (1944). The interference phenomenon has important practical applications (Henle, 1950); thus it must be borne in mind in evaluating the immunizing powers of inactivated vaccines (Andrewes & Elford, 1947) and in safety testing of inactivated vaccines (Henle & Henle, 1944). The masking effect of residual live virus by excess of ultraviolet-inactivated virus was revealed during the course of work in which the rate of inactivation of Rift Valley fever virus (RVF) by ultraviolet radiation was studied. Undiluted irradiated virus suspension appeared non-infective but the same material at higher dilution produced fatal illness in test animals. It was of particular interest that the illness elicited in some of the animals with such partially inactivated virus was markedly different to that produced by the untreated virus.

METHODS

Virus. The pantropic strain of Rift Valley fever virus (RVF virus) obtained from Onderstepoort Veterinary Laboratories was used for irradiation. This virus was obtained from naturally infected sheep and has been maintained by continuous passage in mice. Mice are highly susceptible to this strain on subcutaneous, intramuscular, intraperitoneal, intravenous and intracranial inoculation. Depending on the concentration of virus, signs appear within 18–36 hr. after inoculation and the mice die within a few hours after the onset of signs. At autopsy the chief lesion found is a massive hepatic necrosis. High titre virus suspensions can be obtained from the blood, liver and spleen. For this investigation, heart blood collected from sick mice served as source of virus. The blood was defibrinated by shaking with sterile glass beads and then centrifuged to remove red cells and stroma. The supernatant serum diluted with an approximately equal volume of physiological saline was used for ultraviolet irradiation.

The neurotropic variant of this virus (NRVF virus) was received from Onderstepoort Veterinary Laboratories. It is characterized by its inability to produce symptoms after parenteral inoculation into mice. It elicits characteristic neurological manifestations 3–4 days after intracerebral inoculation in a dose of 0.03 ml. Death occurs in 1–3 days after the onset of signs.

Irradiation. The material was irradiated in quartz tubes with external and internal diameters of 16 and 14 mm. respectively. During irradiation these tubes, held in a horizontal position, were rotated mechanically at a constant rate of 36 rev./min. A standard Hanovia lamp (Arc Tube No. S.B.P. 45525 operated on a supply of 220 volts a.c.) was used as a source of ultraviolet radiation, the vertical distance between the lamp and the centre of the quartz tubes being 25 cm.

Virus titration. Tenfold dilutions were made in a solution of 10% (v/v) normal rabbit serum in physiological saline. Each dilution was used to inoculate six or more 3–4 weeks' old mice, the inoculum being given intraperitoneally in 0.2 ml. dose in the case of the pantropic strain of RVF virus and intracerebrally in 0.03 ml. dose in the case of the neurotropic variant (NRVF virus). The mice were observed for 3 weeks, after which the survivors were discarded or in some cases challenged with active virus. Virus titres (LD₅₀ doses/ml.) were calculated according to the method of Reed & Muench (1938).

RESULTS

Irradiation of pantropic Rift Valley fever virus

In a typical experiment samples of a suspension containing $10^{9.3}$ LD₅₀ doses/ml. were irradiated for periods of 40–60 min. Each sample was injected into groups of mice in dilutions of 10^0 to 10^{-2} or 10^{-3} . Table 1 shows that with the sample irradiated for 40 min. only slight interference was observed, as evidenced by slight prolongation of the incubation period in the mice which had received the most concentrated inoculum. The material irradiated for 60 min. elicited

Table 1. *Inoculation of mice with ultraviolet-irradiated pantropic Rift Valley fever virus*

		Titre of material before irradiation 10 ^{8.3} LD 50 doses/ml.																					
Duration of ultra-violet irradiation (min.)	Dilutions of irradiated RVF virus inoculated	Record of mice inoculated (Days after inoculation)																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
40	10 ⁻¹	—	D
		—	D	
		—	—	D	
		—	—	D	
		—	—	—	D	
		—	—	—	—	—	—	—	D	
	10 ⁻²	—	D	
		—	D	
		—	D	
		—	D	
		—	D	
		—	D	
	10 ⁻³	—	D	
		—	D	
		—	D	
		—	D	
		—	D	
		—	D	
50	10 ⁰	—	—	—	D		
		—	—	—	D		
		—	—	—	—	—	—	N, D		
		—	—	—	—	—	—	N, D ¹		
		—	—	—	—	—	—	—	—	—	—	—	—	N ²			
		—	—	—	—	—	—	—	—	—	—	—	—	—	N ³			
	10 ⁻¹	—	—	D		
		—	—	—	—	—	—	N, D ⁴		
		—	—	—	—	—	—	—	N ⁵		
		—	—	—	—	—	—	—	—	N ⁶		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	10 ⁻²	—	D		
		—	D		
		—	D		
		—	D		
		—	D		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
60	10 ⁰	—	—	—	—	—	—	—	—	—	—	—	—	—	—	— ⁷		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
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		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	10 ⁻¹	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	N ⁸	
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
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		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
	10 ⁻²	—	D		
		—	D		
		—	D		
		—	—	D		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		

D=death; —=survival; N=neurological involvement. Superscript numbers 1-8 refer to individual mice (see Table 2).

symptoms in only one of the mice which received the two most concentrated inocula, whereas four of the six animals which received more dilute material developed typical fatal illnesses. Material which had been irradiated for 50 min. produced an intermediate effect in that the more concentrated inocula elicited delayed illness. What is more striking, however, is that the signs shown by most of these animals were typical of those normally elicited by the neurotropic variant of the virus administered intracerebrally. Although the latent period before the onset of illness was more prolonged than is usual in the case with the neurotropic variant (NRVF virus), the signs were clearly those of neurological involvement.

The type of virus present in the brains and the organs of seven of the mice which showed these atypical delayed signs were determined by inoculation of emulsions intraperitoneally into fresh mice. Table 2 shows that virus was

Table 2. *Distribution of pantropic River Valley fever virus found in mice 1-8 of Table 1*

Mouse number	Deaths recorded when inoculated with emulsion of				
	Brain	Blood	Liver	Spleen	Heart
1	6/6	—	6/6	—	—
2	5/5	0/5	0/5	5/5	0/5
3	5/5	0/5	0/5	5/5	0/5
4	6/6	—	0/6	—	—
5*	5/5	—	—	—	—
6	5/5	—	0/5	—	5/5
7	0/5	0/5	0/5	0/5	0/5
8	5/5	0/5	0/5	1/5	0/5

* Titre in brain $10^{7.2}$ LD 50 doses/ml.

— = not tested.

recovered from all seven of these mice. In all seven, virus with the infective properties of the pantropic strain was present in the brain. In none of those in which the appropriate test was carried out, was virus present in the blood. Virus was recovered from one liver of the six tested, three of the three spleens tested and one of the four hearts tested. A titration of one of the brain emulsions showed virus to be present in fairly high concentration ($10^{7.2}$ LD 50 doses/ml.).

Virus was not recovered from the tissues of a mouse which had received the most concentrated inoculum of material irradiated for 60 min. and which had not shown signs of infection during a 14-day period of observation.

Interference in the central nervous system with the neurotropic variant of Rift Valley fever virus

Interference was also demonstrated with the neurotropic variant (NRVF virus). For these experiments, use was made of mixtures of minimal infective doses of neurotropic variant with large excesses of virus which had been inactivated by 90 min. exposure to ultraviolet radiation. The inactive virus was in the form of brain suspension which had been previously titrated for infectivity, then irradiated and subsequently tested for the absence of

residual virus detectable by intracerebral inoculations. Table 3 shows that a large excess of inactivated virus interfered with minimal infective doses of the live neurotropic variant. Interference was shown with a mixture in which the ratio active:inactivated infectious units was of the order of $1:1.3 \times 10^6$.

Table 3. *Interference in central nervous system on intracranial inoculation of mice with a mixture of active and ultraviolet-inactivated neurotropic variant of Rift Valley fever virus*

Composition of mixture inoculated		Deaths recorded	
No. active infectious units/inoculum	No. inactivated infectious units/inoculum	After intracranial inoculation of mixture	Control* (active only)
1.6	2,100,000	0/5	3/5
1.6	210,000	2/5	.
1.6	21,000	4/5	.

* The control and the three mixtures all contained the same amount of active virus.

Table 4. *Distribution of immunity in mice which survived inoculation with ultraviolet-irradiated Rift Valley fever virus*

Titre of material subjected to ultraviolet irradiation = $10^{8.4}$ LD 50 doses/ml.

Ultraviolet-irradiated RVF virus inoculated originally			Recorded deaths of survivors challenged by intraperitoneal injection of 100 LD 50 doses of pantropic RVF virus
Duration of ultraviolet irradiation (min.)	Dilutions of irradiated RVF virus inoculated	No. deaths recorded	
30	10^{-1}	6/6*	.
	10^{-2}	6/6*	.
	10^{-3}	1/6	4/5
50	10^0	1/6	0/5
	10^{-1}	0/6	3/6
	10^{-2}	0/6	5/6
	10^{-3}	0/6	6/6
60	10^0	0/6	0/6
	10^{-1}	0/6	1/6
	10^{-2}	0/6	6/6
	10^{-3}	0/6	6/6
90	10^0	1/6†	2/5
	10^{-1}	0/6	6/6
	10^{-2}	0/6	6/6
	10^{-3}	0/6	6/6

* One mouse out of each group died as result of late neurological involvement.

† Non-specific traumatic death.

Immunity in surviving mice

The mice which had survived intraperitoneal inoculations of ultraviolet-irradiated pantropic RVF virus, were challenged 3 weeks later by intraperitoneal injection of active pantropic RVF virus. Many of these mice were found to be immune. The distribution of immunity in these groups of mice in a typical experiment is shown in Table 4. From the results it is apparent that

those mice which had received undiluted irradiated virus were immune to the challenging dose. More dilute inocula of irradiated virus gave rise to progressively less immunity. Furthermore, prolonged irradiation (90 min.) decreased the immunity-conferring property.

DISCUSSION

To explain these experimental results it must be assumed that ultraviolet irradiation of RVF virus suspensions in mouse serum under appropriate conditions leads to the inactivation of the bulk of the virus. This leaves only a small amount of virus capable of multiplication and the inactivated virus is capable of interfering with this residuum of live virus.

It is particularly interesting that some of the mice which received ultraviolet-irradiated virus succumbed to an illness characterized by a long incubation period and predominantly neurological manifestations. At first it seemed that this might be explained simply by interference which occurred more readily in the liver than in the central nervous system. Mice which received the irradiated virus would then die of an involvement of the central nervous system which, during the course of the normal illness in mice, had no time to develop. Interference can, however, be demonstrated also in the central nervous system by using appropriate mixtures of active and ultraviolet-irradiated virus injected intracerebrally. It seems more likely that the phenomenon is dependent, at least in part, on differences of tissue affinity for the virus. An organ such as the liver can be regarded as having a high affinity for the pantropic strain whilst the affinity of the central nervous system for the same virus is less. When, therefore, partially inactivated virus is inoculated, the liver takes up most of the virus. If most of the virus is inactivated this will interfere with the small amount of live virus present. Most of the liver and other highly susceptible tissues will be spared from infection by live virus, and will remain protected against the virus produced from the limited number of cells initially infected by it. Virus resulting from the first growth cycle, which occurs in the few liver cells infected with live virus, is released into the circulation before antibody forms. The central nervous system, which by its lesser affinity has not so readily absorbed either inactivated or active virus, will lack the protection given by inactivated virus and will remain susceptible to such newly produced virus. The animal therefore succumbs to delayed neurological involvement instead of to a rapid hepatic involvement as occurs normally.

The solid immunity which develops in mice which have received a single dose of irradiated virus in high concentration probably also depends on residual virus which is capable of multiplication. When the proportion of inactivated virus is overwhelming and the amount of residual live virus very small as in the case of the material irradiated for 60 min. (Table 1), the amount of virus which undergoes multiplication is limited. The amount of virus formed is too small and the speed of its release is probably too slow to elicit clinically recognizable disease, whether neurological or hepatic, before

a significant degree of immunity becomes established. With virus suspensions which have received the appropriate dose of radiation, a temporary or perhaps even permanent infection is established which initially is kept latent by the presence of interfering inactive virus and later by increasing cellular and humoral immunity. When mice receive inocula of RVF virus which has been subjected to more prolonged irradiation, the survivors do not show the same solid immunity. In this case 'loss of antigenicity' is presumably due to complete inactivation of the virus.

The establishment of a permanent latent infection as the basis for a solid immunity which follows a virus disease has been postulated on several occasions. It is possible that in the case of the experiments with RVF virus here reported an additional factor is the production by ultraviolet-irradiation of virus so altered that it is no longer fully pathogenic but capable of establishing a permanent latent infection. It is well known that the herpes virus may establish a latent or inapparent infection which is associated with a persistence of antibodies and immunity to re-infection. The establishment of permanent latent infections in immunization against a virus may be a much more general phenomenon than at present recognized. We hope that the phenomena described in this paper may be a starting-point for attempts to detect the presence of latent infections in actively immunized animals.

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ADDENDUM

A series of papers on Rift Valley fever virus by Mims (Mims, C. A., 1956, *Brit. J. exp. Path.* **37**, 129) were seen after this paper had been completed. Mims describes a similar interference effect due to incomplete virus. He finds that when mice are inoculated with material containing high concentrations of incomplete virus, the mice may survive and develop immunity or late neurological involvement may occur. In the latter case virus is found in the brain but not in the liver.

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ALBERT JAN KLUYVER

(Facing p. 499)

Obituary Notice

ALBERT JAN KLUYVER, 1888–1956

‘On the one hand, the friend who is familiar with every fact of the story may think that some point has not been set forth with that fullness which he wishes and knows it to deserve; on the other, he who is a stranger to the matter may be led by envy to suspect exaggeration if he hears anything above his own nature.’

From Pericles’ Funeral Oration; Thucydides:

The Peloponnesian War; Crawley translation.

In 1895, when Albert Jan Kluyver was just beginning his formal education in a grammar school, the Netherlands Government established a small laboratory for general and applied microbiology at the Polytechnical School in Delft. It was created specifically in order to provide for a fuller development of the potentialities of a brilliant young scientist, M. W. Beijerinck, whose position as microbiologist at the Netherlands Yeast and Alcohol Manufactory did not allow him sufficient scope. The curriculum of the Polytechnicum did not then include any biology, and for lack of a more appropriate allocation, the new institute was incorporated in the chemistry department. When, ten years later, the Polytechnical School was elevated by Government action to the rank of Technological University, and Kluyver enrolled there as a first-year student, microbiology was left attached to the division of chemical technology; it has remained there to this day.

Now this fact did not influence Beijerinck’s activities to any great extent. The investigations carried out by himself and his collaborators have stressed general biological rather than chemical aspects of microbial behaviour. What was accomplished during the quarter century of Beijerinck’s directorship need not concern us here; it has been admirably reviewed in the great three-part biography, published in 1940, in Volume VI of Beijerinck’s *Collected Works*. Suffice it to say that at the time of Beijerinck’s retirement, in 1921, the Delft laboratory for microbiology had acquired world-wide renown.

On 18 January 1922, the high functionaries of the Technological University ceremoniously assembled in the concert hall of the city of Delft. In those days the university did not yet possess an auditorium, and the city concert hall was often used for this purpose. The occasion for the solemn gathering, attended also by students and other interested persons, was that on that day the newly appointed successor to M. W. Beijerinck, Dr A. J. Kluyver, was to deliver his inaugural address (1).

Kluyver had completed his studies for the degree of chemical engineer in 1910, with an outstanding record. By then the chemistry division had been expanded to include another branch of biology; in 1907 Beijerinck’s eminent pupil, Dr G. van Iterson, jun., had been appointed Professor of Microscopical

Anatomy. And Kluyver, immediately after his graduation, had accepted a position as van Iterson's assistant, a post he had held until 1916 with two interruptions, caused by a period of study with Hans Molisch, the famous Viennese botanist-chemist, and a spell of service in the Dutch army. During the first four years of his assistantship he had not only successfully carried out his many arduous duties, but in addition he had assembled an impressive mass of observations on sugar fermentations by various yeasts. These formed the basis for a dissertation on biochemical sugar determinations (2) which, in 1914, had earned him the degree of D.Sc. 'with distinction', the highest recognition of superior merit that the Technological University can bestow.

Meanwhile, van Iterson had become deeply impressed with the scientific ability and personal characteristics of his young collaborator. He had recommended him for the position of consultant for the Dutch East Indies to the Department of Agriculture, Industry and Commerce, and remained in close touch with him during Kluyver's sojourn in the colonies. And when the problem arose of finding a promising future occupant of the chair which Beijerinck had to vacate on account of having reached the age limit, van Iterson had built up a strong case in favour of his former assistant. Owing to his many-sided and prominent activities, van Iterson's voice had become very influential in scientific circles, and his advice, always carefully thought out, ably documented, and forcefully presented, was generally heeded. Thus it had come to pass that, in 1921, the Netherlands Government had appointed A. J. Kluyver as Beijerinck's successor, thereby completing the preliminaries to the inaugural address.

The new professor of general and applied microbiology, then 33 years old, began his lecture by developing the theme that in the not too distant future the human race would be facing a serious problem resulting from the depletion of the limited supply of fossil fuels, the main source of energy for the world's rapidly expanding industry. The speaker argued that eventually other sources of energy would have to be tapped, and with almost prophetic foresight he predicted that man might some day be in a position to utilize effectively the enormous intra-atomic energy that Rutherford's studies had revealed. But another solution was considered at greater length, not only because it seemed a more immediate one, but also because it served the purpose of showing that the application of micro-organisms on an industrial scale could render significant services in this respect. As an example the manufacture of alcohol by fermentation of carbohydrates was cited as a potential means of supplying fuel for internal combustion engines; furthermore, the war-born industries producing glycerol, butanol and acetone, fats and proteins from cheap and readily available sources of agricultural raw material through microbiological processes were discussed in some detail, as well as additional possibilities, such as the large-scale production of lactic, citric and fumaric acids with the aid of microbes.

In fact, the major part of the inaugural address was devoted to a defence of the thesis that the technological potentialities of microbiology fully justified the inclusion of this subject in the curriculum of the Technological University.

Not until the very end of the lecture did Kluyver stress that he did not wish to leave his audience with the impression that he would neglect the teaching and development of general or theoretical microbiology, and that he did not want to be counted among those who are apt to consider knowledge as attractive only if it can contribute to our material well-being. The arguments in favour of the theoretical aspects of the science he was to represent were still based in part on his conviction that an ever-increasing knowledge of the properties of micro-organisms is a prerequisite for their eventual application; but in addition he made the significant statement that he, too, was far from insensitive to the fascination which the study of nature and the struggle to penetrate into her tenaciously guarded secrets holds for the investigator. Nevertheless, the concluding remarks were too general to determine what possibilities the speaker envisaged for developing this part of his programme.

Viewed in retrospect this is not surprising. For we now know that Kluyver, when he finally and after much hesitation had accepted the appointment as Beijerinck's successor, had himself barely begun to be a serious student of microbiology. No wonder, therefore, that he felt diffident when he had to face the colleagues of his great predecessor, and that he restricted his remarks largely to those aspects of microbiology that had already borne fruit, or that could reasonably be expected to contribute more or less immediately to technology. It was a safe approach, and in this way he could also hide his embarrassment in having to appear on the rostrum as an expert microbiologist. In later years he occasionally reflected on the fears and trepidations he experienced during those early days, and admitted that he spent the few months preceding the inaugural address in frantic and anxious perusal of microbiological literature, trying to absorb as much as was humanly possible in the short time available before he had to face his audience.

Those must indeed have been difficult times for him. For the inaugural address was merely a prelude; anon he had to start giving lectures in the field in which he had had very little experience. And this was still easy compared with the task he had to face when, as soon happened, new students appeared at his laboratory with the intent of specializing in microbiology, and he had to guide their researches. The difficulties may not have been evident to the students; they were probably unaware that the daily conferences with the professor served as much to fan their own interests, and to suggest new experiments, as to acquaint the director with the behaviour of micro-organisms through taking notice of the latest results, so that in this manner he could most effectively increase his own knowledge.

During this phase he could also lean heavily on his two capable assistants, L. E. den Dooren de Jong and H. J. L. Donker, whom he had inherited from Beijerinck. Especially the former, who had studied with Beijerinck for several years, seems to have made his much greater experience in microbiology available to the new professor in a very effective manner, and Kluyver often acknowledged the debt of gratitude he owed him for the support so unstintingly given.

Within a year a great deal had been accomplished. Aided by his enormous

capacity for assimilation of factual knowledge, his highly retentive memory, his fine critical ability, and his strong desire for integration, Kluyver had sufficiently familiarized himself with various problems so that he could perceive in what directions the most important developments could be expected. But of even greater significance for this development was his high sense of responsibility which caused him to adopt a strict regime of work from early morning till midnight and after, with little more diversion than an hour's walk with a close friend on Sunday afternoons. Reflecting afterwards on his qualifications at the time of his appointment, he seems to have concluded that the will to learn coupled with a profound sense of duty was even more important than extensive knowledge of a subject, because the latter could always be acquired by intensive study.

Thus Kluyver was soon in a position where he could draw upon his rapidly increasing knowledge and comprehension in order to guide the work of the students in an admirable manner. The daily interviews continued, and their charm was considerably heightened by their utterly informal nature. This contributed much to establishing a personal contact with the students, and to creating in the laboratory a spirit very different from that so often encountered in European institutions of higher learning, where the relationship between professor and students resembles that between the lord of the castle and the lowly tenants in a feudal society. The discussions were held as between equals, with common interests and striving to learn together; they were frequently interspersed with a number of recurring phrases, such as: 'some day we'll know', 'keep smiling', 'never say die', intended to make light of the difficulties experienced and to encourage the struggling novices. And with the example set by the director, the enthusiasm of the students was maintained at a high level, and work towards the solution of a problem became a joy, not to be terminated at 5 p.m., when the personnel went home and the door was locked, but continued throughout the evening. The assistants, who had keys, were always ready to let students in or out after closing hours; and if they, too, had left, entrance to the laboratory could always be obtained through the director's home and study, adjoining the laboratory. It has happened that during the early morning hours the police would ring the housebell in order to announce that someone evidently had forgotten to turn off the lights in the laboratory, only to be told by the director that this was not the case; that, in fact, work was still going on, and might well continue for some hours more.

During the second year of Kluyver's directorship, when he could recognize aberrant results, a chance discovery was made which was to exert a profound influence on the trend of research in the Delft laboratory. On a yeast extract + glucose + calcium carbonate agar plate, streaked with a suspension of bacteria that had grown in a flask of beer exposed to air, colonies developed that initially caused a complete dissolution of the carbonate in their vicinity. This was not unexpected; the beer culture was meant to yield acetic acid bacteria, and these were known to produce gluconic acid from the sugar. The cleared areas around the colonies would thus result from a secondary reaction

of the acid with the carbonate. But in this case a novel feature was observed; in the cleared areas large crystals, evidently of a calcium salt, subsequently appeared. Analysis of these crystals showed them to be composed of calcium 5-keto-gluconate.

This discovery gave the impetus to a comparative study of the properties of various types of acetic acid bacteria. The results led to the inference that this group of organisms effects a stepwise oxidation of glucose, through gluconic and ketogluconic acids, and that, depending upon the oxidative capacity of the particular representative, different oxidation products would eventually accumulate. Thus the types of acetic acid bacteria could be arranged in order so that successive members of the series indicated their greater oxidative capacity by their ability to oxidize the end products formed by the preceding ones (3).

The properties of the newly isolated type, designated as *Acetobacter suboxydans* to emphasize its low oxidative capacity, further suggested that it might generally cause an incomplete oxidation of many hydroxylated compounds. Pertinent experiments soon showed that a number of sugar alcohols were indeed oxidized to the corresponding keto-derivatives in well-nigh quantitative yields. This emphasized the difference between *A. suboxydans* and Bertrand's sorbose bacterium, *A. xylinum*, because the latter, although performing similar incomplete oxidations, subsequently oxidizes the products further. Lacking this capacity, *A. suboxydans* thus revealed itself as an 'ideal sorbose bacterium' that should prove useful for the manufacture of many keto-compounds. This potential application was patented somewhat later, showing that Kluyver was aware of the implications of this study, and not averse to thinking in terms of applied microbiology. It may be remarked that, following the development of the Reichstein synthesis of vitamin C, in which the production of sorbose from sorbitol is an important step, this conversion has generally been accomplished on a commercial scale with cultures of *A. suboxydans*.

Of far greater importance than the commercial application of this organism was, however, the effect these studies had on the development of a programme of the Delft institute. They had shown that chemical investigations can contribute to a better understanding of the behaviour of micro-organisms, and, contrary to Beijerinck, Kluyver had an intrinsic interest in biochemical problems. This had started during his work on sugar determinations with the aid of various yeasts; he had then familiarized himself with the advances made in the interpretation of alcoholic fermentation due to the introduction of the notion that this process can be considered as the end result of a series of consecutive step reactions (4). Now it may be true that the mere knowledge of a subject is apt to engender a continued interest in its development. But rarely does this lead to contributions as far-reaching as those made by Kluyver during the next five years.

The programme that gradually took shape in Kluyver's mind was outlined in a lecture he delivered before the Netherlands Chemical Society early in 1924 (5). As a subject he had chosen the metabolic activities of micro-organisms; it was discussed from two angles. The first and greater part of the

paper contained a general survey of the enormous diversity in substrates utilizable and products formed by different types of microbes. The second part represented an attempt to discover among this diversity some unifying principles; it dealt with energetic and chemical considerations, respectively. In line with the then current ideas, the catabolic and anabolic aspects of metabolism were sharply differentiated, and it was pointed out that the former, however different their specific manifestation, all shared the property of releasing energy. This had led to the concept that catabolism provided the energy with which the anabolic processes were accomplished. Now it is noteworthy that, even at this time, Kluyver perceived that such a concept of energetically linked reactions left unsolved the important problem of the nature of the energetic linkage, especially because it seemed that the two phases of metabolism did not have any components in common. This problem was posed, though not answered, in the passage: 'Hence I should like to ask the physical chemists among my audience whether they know of similar cases of coupled reactions in inanimate systems, and in how far they would have fundamental objections against accepting the occurrence of such energetic coupling.'

The last part of the paper was concerned with speculations on unifying principles of a chemical rather than of an energetic kind to account for the various catabolic processes themselves. These centred around the recent studies with *Acetobacter suboxydans*. It was argued that the degradation of the substrate, supposed to proceed step-wise in fermentation, could now be seen to follow a similar path in oxidative metabolism as well, and that the discovery of a series of organisms that can oxidize glucose to a different extent might help in gradually acquiring a better understanding of the step reactions proper.

It is perhaps not superfluous to stress that at this time very little was known about oxidative metabolism, and that even the interpretation of fermentative processes barely extended beyond the recognition that these represent sequences of step reactions. Together with the utter lack of indications that enzymes involved in oxidative metabolism could be isolated, this permits us the better to appreciate Kluyver's delight in having found a possible approach to its analysis through the use of micro-organisms. And the avowed implication was that such an analysis would also apply to the interpretation of oxidative metabolism in higher organisms.

Meanwhile, the number of students in Kluyver's laboratory had increased; hence it became possible to engage in a more detailed investigation of various metabolic processes carried out by specific groups of microbes. In a very few years these studies had yielded results that illustrated the applicability of the notion that all catabolic reactions are composites of series of step reactions. Furthermore, they had led to the conclusion that the step reactions themselves were essentially similar, and that each one could be represented as a special case of hydrogen transfer from a donor to an acceptor molecule. The great diversity in end products was thus reduced to differences in the donors and acceptors participating in the step reactions.

First published in a preliminary form in 1925 (6,7,8), this concept was fully elaborated in an extensive publication in which the principle of hydrogen transfer was used to account for the enormous variety of the known biochemical phenomena (9). At the end of this paper the idea was advanced that even anabolic processes are interpretable along the same lines. This theme was the subject of a later article in which the similarities and relationships between fermentation, oxidation and anabolic reactions were discussed (10). Here the thesis was proposed that the connexion between catabolism and anabolism should no longer be sought in energetically-linked reactions that have no material component in common, but that it seemed far more reasonable to look upon catabolic reactions as a means of producing, as intermediate products, special chemical entities from which anabolic reactions can proceed spontaneously. The latter could then be regarded as series of simple, consecutive step reactions that, just as the catabolic ones, do not require an external energy supply because the participating molecules are themselves at a sufficiently high energy level.

In order to appreciate fully the immense clarification brought about by these contributions, and the superbly logical approach on which they were based, these developments must be viewed in their proper perspective. Only those who are familiar with the general status of biochemical knowledge around 1925 will understand the magnitude of the advances made.

It should also be realized that the force of the argumentation derived as much from the synthesizing ability of Kluyver's mind as from his vast knowledge of the enormous variety of biochemical reactions encountered among the micro-organisms. His interest in the diversity had by no means subsided; but it had gradually become subservient to the philosophically more engrossing search for general principles. By including numerous examples of anaerobic and aerobic metabolic types characteristically restricted in their occurrence to the microbial world in developing the concept of hydrogen transfer as the central and common feature of all metabolic activities, Kluyver extended its fruitfulness far beyond achieving a synthesis of the seemingly irreconcilable views of Wieland and Warburg concerning the mechanism of oxidative processes, independently and simultaneously advanced by Fleisch and by von Szent Györgyi. This also explains the great impact that Kluyver's publications have had on biochemical thinking ever since 1926.

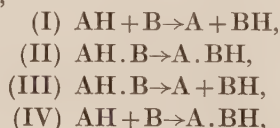
In less than a decade the programme Kluyver had outlined in his 1924 lecture had thus produced results that exceeded even the highest expectations. Now a beginning could be made with the accumulation of additional material, consciously selected to fill gaps in our knowledge, or to check apparent inconsistencies. In this manner originated the many monographs, usually theses for the doctorate degree, prepared by Kluyver's collaborators, (e.g. (11-21)). These are more than just biochemical studies; each one contains descriptions of adequate elective and pure culture techniques that make representatives of the particular groups of bacteria more readily accessible. Besides, the general morphological and physiological properties of various members of these groups

were ascertained, and this in turn led to a recognition of the important distinguishing characteristics of representative clones.

These dissertations also contain a wealth of information on methods for the qualitative and quantitative analysis of metabolic products. This aspect was appreciated, for example, by Marjorie Stephenson, who, in her contribution to the Kluyver Jubilee Volume of 'Antonie van Leeuwenhoek', published on the occasion of Kluyver's quarter century professorship, wrote: '...so ably and thoroughly was the analytical work carried out that this series of theses still remains the most reliable source of quantitative data to which workers on bacterial fermentations may turn' (22), p. 34).

During the early years it was especially a variety of sugar fermentations that was studied in Kluyver's laboratory. The reason for this choice is simple; several different patterns of such fermentations were known to exist, the decomposed substrate could be recovered largely in the form of easily determinable products, and the quantitative relationships between them could thus be established. The relatively insignificant amount of cell material formed during the growth of the cultures could generally be neglected without endangering the balances in which the amount of substrate decomposed could be equated with the quantity of products recovered. These balances were not only a means of checking the possibility that some quantitatively important product had been overlooked; in addition, they were used to develop ideas on the nature of the specific step reactions which could then be further explored by pertinent experiments. They also revealed the dependence of the relative proportions in which the fermentation products are formed on environmental factors, often implicating the presence or absence of specific alternative hydrogen donors and acceptors, and thus emphasized the dynamic nature of the processes and the interplay between various reactions which the intermediate products can undergo.

The enunciation and elaboration of the concept of hydrogen transfer as the fundamental characteristic of all metabolic processes led the University of London to invite Kluyver for a series of lectures. These were delivered in May 1930, and published the following year by the University of London Press (23). It was here that the term 'comparative biochemistry' was first launched, in the following passage: 'A study of the chemical activities of micro-organisms reveals all the advantages which may be derived from "comparative biochemistry". Although this line of study has not as yet been much developed, it may in future win the same significance for biochemistry as "comparative anatomy" has already long ago attained for anatomy.' Here, too, the now so familiar reaction equations,



were first presented as a summary of the essence of biochemistry.

The pattern of this series of lectures, which starts with a general survey of metabolic types found among micro-organisms, and proceeds to a discussion

of oxidations by *Acetobacter suboxydans*, to an analysis of the fundamental features of various fermentations, and finally to a consideration of anabolic reactions in the light of previously developed ideas, follows closely the sequence in which Kluyver's outlook had gradually evolved. The same pattern can be recognized in his later publications of a general nature, including the recent Prather lectures, given at Harvard University in 1954, and published in 1956 by the Harvard University Press (24). The logical progression by which the ultimate concept of the unity in biochemistry was shaped cannot be illustrated more clearly.

Some 25 years have passed since the London lectures were delivered. If we compare the reaction equations there proposed with those currently used to describe the individual step reactions and their interrelations in metabolic events, it is clear how vastly our knowledge of biochemical mechanisms has increased. The earlier proposed equations now look rather primitive, and hardly any of the key intermediate products then evoked are seriously considered to-day. Kluyver himself has been fully aware of these developments, and has frequently reviewed them. But, as he cogently argued in the Harvard lectures, they have not in any way invalidated, in fact, they have rather substantiated the essential validity of the earlier concepts. A sound evaluation of the method by which man increases his comprehension requires the recognition that science represents a gradual approximation to the interpretation of natural phenomena, and that it is the grand generalizations that mark the fundamental advances. Kluyver's contributions to biochemistry are of the latter quality; above all he was a philosopher interested in details only in so far as these could contribute to the development of fundamental principles.

This is also illustrated by the studies on oxidative metabolism carried out in his laboratory. In the early 'thirties a beginning was made with an experimental attack on mould metabolism; an incentive may have been the consideration that the microbiological production of, for example, citric, kojic, fumaric, and other acids on an industrial scale would benefit from a better understanding of the mode of formation of these substances than was available at the time.

Although it had been established that environmental conditions may exert a profound influence on the nature and quantity of incomplete oxidation products formed in mould cultures, the reasons for the observed variations had not been sufficiently elucidated. Now, in the first experiments on kojic acid formation it was found that even under seemingly identical conditions the results obtained in parallel cultures fluctuated considerably. Owing to this complication a new methodology was introduced into microbiological research, to wit, the cultivation of filamentous micro-organisms in continuously agitated liquid media; it was the result of Kluyver's cogitations on the reasons for the unexpected differences in duplicate experiments.

In a clear and cogent manner the reasons for abandoning the commonly used stationary cultures were set forth in the first of a series of publications on mould metabolism (25, 26, 27). It was pointed out that reproducible results can only be expected from studies with identical material under fully comparable

conditions, and that this requirement was not fulfilled by stationary cultures. Here the organism develops partly as aerial mycelium at the surface, where the concentration of oxygen is high, and that of the substrate low; partly as submerged growth in the liquid, where the conditions are just the opposite. Because the rate of development is apt to be very different under such diverse conditions, and because the latter will also affect the enzymic activity and composition of the cells, it became obvious that, as long as cultures composed of different types of cell material, grown under different conditions, and exposed to different environments were used, uniform results would rarely if ever be obtained. The following passage, translated from this paper, may be quoted to show the succession of arguments leading to a rejection of the stationary culture method for studies aimed at analysing details of mould metabolism:

If this is kept in mind it is no longer surprising that different results are so often obtained in duplicate cultures under presumably identical conditions. But even if such cultures were to show satisfactory agreement it is still impossible to specify sharply the conditions responsible for a particular direction of the metabolic processes. If, for example, a certain situation leads to the production of large amounts of citric acid it is impossible to decide which of the cells in the mycelium, developing under heterogeneous conditions, have been responsible for the acid production. And even if it were possible to determine which cells have produced the acid, and to specify the optimum conditions for this process, the result would be of only restricted significance, for it would be erroneous to conclude that any cell of the organism would display the metabolic activities characteristic of the acid production under these optimum conditions. One can find many examples in the literature to show that the metabolism of mould cells is greatly dependent on the conditions under which they were grown, and we shall presently add further proof for this contention. Now it is obvious that these conditions are quite heterogeneous for the cells developing in a mycelial mat on a liquid medium. In summary we may therefore conclude that one has to reckon with the presence in such a mat of cells of heterogeneous origin, exposed to heterogeneous conditions. It will then be clear that one can obtain at best a very limited picture of the metabolic processes of moulds with the aid of the usual stationary cultures. Consequently a deeper penetration into the problems of this metabolism is possible only if it is investigated with cell material of homogeneous composition, studied under homogeneous conditions (23), p. 70).

Thus it became necessary to search for a method whereby such cell material could be procured. This was achieved by growing moulds in liquid media with continuous agitation. The organisms then do not produce any aerial mycelium, but develop in the form of small balls of tangled threads, composed of cells grown under conditions as nearly homogeneous as possible. With the aid of this technique satisfactory agreement between replicate experiments was soon obtained, after which a study was made of the influence exerted by specific factors on the products formed.

That the development of the submerged culture technique has also been of decisive industrial importance will be acknowledged by any one who is familiar with current manufacturing procedures in which moulds and actinomycetes are used. It would now be difficult to conceive of a large-scale production of penicillin or streptomycin, for example, without the use of submerged, aerated

cultures. But that this method was devised some 25 years ago in Kluyver's laboratory, on purely theoretical grounds, is, I fear, still insufficiently appreciated.

A few years after these studies on mould metabolism Kluyver turned his attention to other problems of oxidative metabolism, this time with resting cell suspensions of bacteria. The discovery by Cook & Stephenson (28) that such suspensions may cause a complete disappearance of added substrate with only partial recovery in the form of the normal oxidation products, CO_2 and H_2O , was the impetus to these studies. The findings of Cook & Stephenson had meanwhile been extended by Barker (29), who had shown that in such cases a hitherto unexpected degree of assimilation occurs. Within two years after Barker's publication appeared, the studies by Giesberger and Clifton in Kluyver's laboratory had revealed the generality of the phenomenon, and the possibility of preventing the assimilatory reactions by adding dinitrophenol or azide to the cell suspensions (30,31,32). At first these results seemed to hold out promise for significant developments in our understanding of assimilatory mechanisms, but this has not been fulfilled; the great advances in this field have come from studies with enzyme systems.

Around the same time Barker, studying the methane fermentation in Delft, furnished the experimental proof for the contention that this process can be considered as the result of substrate dehydrogenation with CO_2 serving as the ultimate acceptor (33,34). This investigation was later extended by Kluyver and Schnellen (35,36) who also studied the production of methane from CO and H_2O . This curious fermentation, which can proceed in an atmosphere of pure CO , was shown to proceed in two stages:



and



Not only did these studies create a special interest in the utilization of CO by micro-organisms which recently led Kluyver's collaborator Kistner to isolate CO -oxidizing bacteria (37); they were also the starting-point for Kluyver's ideas on the role of CO_2 in heterotrophic metabolism.

Simultaneously with Barker's demonstration that bacteria can reduce CO_2 to CH_4 , Woods (38) and Wieringa (39) discovered the synthesis of formic and acetic acids from CO_2 and H_2 by *Escherichia coli* and *Clostridium aceticum*, respectively; and Wood & Werkman (40) found that CO_2 is assimilated by propionic acid bacteria. Later work on this phenomenon indicated that CO_2 is used in the synthesis of succinic acid from a three-carbon molecule (41). Contemplating these results, Kluyver realized that even for heterotrophic micro-organisms CO_2 was not merely a metabolic end product; and this, together with some additional information, led him to the generalization, expressed in a lecture delivered in Helsinki early in 1939, that: 'the recent investigations make it highly probable that actually CO_2 is a truly indispensable nutrient for all heterotrophic cells... Although, soon after the beginning of our era, Pliny characterized it as "*spiritus letalis*", there is now, in view of the indispensability of this gas for all living creatures, much more reason to

designate it as "spiritus vitalis" (42), pp. 86–8). At the time of its pronouncement this statement may have seemed rather far-fetched, especially because a satisfactory explanation for a presumed specific function of CO_2 in heterotrophic metabolism had not yet been given. But later work, particularly with isotopically labelled CO_2 , has amply confirmed this bold generalization.

During the first fifteen years of Kluyver's directorship many different bacterial types were used for biochemical and other studies in the Delft laboratory, and Kluyver gradually acquired an extensive knowledge of their properties. Consequently he could begin to pay serious attention to the problem of bacterial classification. Already in the inaugural address he had hinted that this was in an unsatisfactory state, though it is doubtful whether at that time he could have indicated the reasons for the confused situation. But later these became clear; the chaotic condition had to be ascribed to the haphazard manner in which bacterial classification had developed, without the benefit of uniform guiding principles. Bacteriologists with divergent aims and interests had approached the problem with different, sometimes even incompatible opinions as to the relative values of differential characteristics, and the taxonomists had thus become confronted with the well-nigh impossible task of organizing the often conflicting and woefully incomplete information into a coherent system.

Now the fact that it had been found possible to co-ordinate the multitude of biochemical activities of micro-organisms through the formulation of some simple principles raised the question whether it might not be possible to use these latter in an attempt to frame a more rational approach to the problem of bacterial classification as well. *A priori* this seemed promising in view of the outcome of the studies with the acetic acid bacteria which had shown that different representatives of the group can be arranged in order of their respective oxidative capacities, thus permitting a simple and effective differentiation within the group. The application of biochemical criteria on an expanded scale, and with proper attention being given to the newly acquired insight into biochemical processes in general, might therefore be expected to result in the emergence of a system of classification in which taxa of higher rank than species could be defined by a combination of judiciously selected morphological and biochemical characteristics. Such a system would have the advantage of being composed of better integrated units than most of those previously proposed; it should also indicate what combinations had not yet been encountered among bacteria, and in this manner provide the appropriate taxonomic position for such organisms, if discovered.

The outline of such a system was published in 1936 (43); it has had a salutary effect on bacterial systematics, and many of the taxa delineated in this paper have found their way into current literature. And the genus *Kluyvera*, recently proposed by Asai, Okumura & Tsunoda (44), now fills the space reserved to accommodate the polarly flagellated bacteria with a metabolism corresponding to that of *Escherichia coli*; such organisms were not known in 1936.

It is true that the subdivision of bacteria with an exclusively oxidative

metabolism presented difficulties because it was not clear what biochemical criteria could be used for the delineation of taxa comparable to those established among fermentative types on the basis of different fermentation patterns. This has made the genera with oxidative metabolism too unwieldy for present-day comfort. With the increased understanding of the differences in oxidative pathways it may be expected that such large genera as, for example, *Pseudomonas*, could be further split by using this information imaginatively. This would have the advantage that the new units contain fewer species, which would facilitate determinative procedures. This, however, is a task for the future.

Shortly after Kluyver had assumed his position as professor at the Technological University he arranged with Professor Dr Johanna Westerdijk, director of the Central Bureau of Fungus Cultures in Baarn, Holland, to have the collection of yeast cultures maintained in her institute transferred to the Delft laboratory which already possessed an extensive collection of yeasts owing to Beijerinck's interest in this group. The consolidation seemed of mutual benefit because in Baarn the emphasis had shifted more and more to the filamentous fungi whose number was steadily increasing with the result that space and personnel were there at a premium. A small annual grant permitted Kluyver to appoint a special assistant who was charged with the upkeep of the collection, soon to become the finest in the world, and with initiating a comparative study of the numerous strains. The latter developed into an exhaustive survey; the cultures were checked against the original descriptions; properties not before determined were ascertained; and the accumulated information was used to determine whether the large number of species described in the course of time should be perpetuated. The result of this programme was the publication of a series of monographs that represents the solid foundation of current knowledge of the morphology, physiology, and classification of the yeasts (45–48). The importance of these publications for workers in the field can hardly be overrated; in 1941 Henrici⁽⁴⁹⁾ prepared an English review of the first of these monographs comprising no less than 82 pages.

Even though a large number of more or less incidental publications has not been discussed, the preceding pages may have given a fair impression of the wide scope of Kluyver's work. There is, however, one aspect that has not yet received the attention it deserves in view of the influence it has exerted. This is Kluyver's strong advocacy of the use of micro-organisms for biochemical investigations. On many occasions he pointed out that more suitable objects for such studies were difficult to imagine. In the first place they are easily handled, and, as pure cultures, represent material far more homogeneous than most tissues, let alone entire plants or animals. In the second place, the greatest range of metabolic manifestations is found only among the microbes; hence they alone can be used for a study of those extreme biochemical processes that are nevertheless of paramount importance for the development of comparative biochemistry. During the second and third decades of this century this was still insufficiently appreciated, and Kluyver sometimes complained that biochemists, by neglecting the potentialities of the

micro-organisms, impeded the advancement of general biochemistry. But he was gratified that his promptings were more and more heeded, and that gradually biochemists learned to utilize the microbes in accord with his persuasions. The enormous growth of biochemical literature dealing with studies involving micro-organisms testifies to his influence, and the great advances in our understanding of biochemical mechanisms is in no small measure due to this changed attitude. Much of this work is manifestly predicated upon the idea that a more profound understanding of the mode of degradation of a substrate, or of the formation of a metabolic product under the influence of a bacterium, yeast, mould, alga, or protozoan, will contribute equally to our interpretation of similar processes occurring in higher plants and animals, and this is based squarely on Kluyver's concepts of the value of comparative biochemistry.

It has become increasingly clear that the phenomenal progress in biochemical comprehension must be ascribed largely to the introduction of studies with isolated enzyme systems. Yet it is a curious fact that not until very recently have enzyme studies been carried out in Kluyver's laboratory. In order to make this understandable it is not sufficient to argue that adequate methods for obtaining enzymes from microbes were not earlier available. No doubt this may have been a contributory factor. But a more relevant cause for Kluyver's essentially negative attitude towards enzyme studies will now be considered in some detail.

Admittedly, up to 1930 there seemed to be little prospect that enzymes could be studied by chemical methods. It is true that for about a century the conversion of starch to glucose by extracts from seeds and by sputum had been known, and in the course of time other extracts had been obtained that could split oligosaccharides or proteins into their constituent units. Such reactions were ascribed to the activity of specific enzymes present in the extracts, and some biochemists were engaged in attempts at purifying these enzymes in the hope of learning something about their nature and mode of action. In 1926 Sumner had even announced the crystallization of the enzyme that hydrolyses urea to ammonium carbonate. But for many years biochemists were reluctant if not downright unwilling to accept Sumner's great contribution.

I do not know whether Kluyver was among the few who, at an early date, accepted the claim that urease had been crystallized. Even if he had, he would not have attached to it the importance it deserved. For at that time Kluyver felt that the hydrolases had but little bearing on the fundamental biochemical events. Granted that they serve a useful function in converting high-molecular compounds into small, readily diffusible molecules, such processes seemed to him of minor significance compared to the oxido-reductions, the fundamental processes intimately associated with energy provision and growth.

Now it is true that the discovery of zymase had long ago shown that oxido-reductions can also be induced by enzymes, and the subsequent preparation of cell extracts that could cause other fermentations and oxidations had contributed its share to indicate the feasibility of *in vitro* studies of enzymic

reactions other than hydrolyses. But such studies did not appeal to Kluyver, and this for two different reasons.

The first had its origin in the curious result of an investigation of 'co-zymase', conducted in collaboration with Struyk (50,51). Although it seemed improbable that effective methods could be devised for the fractionation and ultimate purification of the unstable 'zymase', an obviously very complex mixture of substances representing most of the contents of yeast cells, Harden's discovery of a diffusible, heat-stable substance, 'co-zymase', that could be eliminated from a zymase preparation of dialysis, and in whose absence the zymase failed to provoke the typical fermentation, held out hope for a more fruitful approach. Kluyver and Struyk therefore embarked on a study of this situation.

A zymase preparation was freed from 'co-zymase' by ultra-filtration; as expected, the residue failed to ferment sugar. But if part of this residue was boiled and added to a non-heated sample, a normal fermentation could be induced. This result was explained by assuming that the coagulated protein in the boiled fraction protected the zymase from decomposition by the proteases also present in the extract.

To my knowledge this experiment has not been repeated by later workers, nor has the unexpected result been satisfactorily explained in the light of present-day knowledge. But it is understandable that it led Kluyver to the conclusion that the 'co-zymase effect' had nothing to do with a specific activity of small, heat-stable molecules, save that they might function as hydrogen acceptor for the initial formation of pyruvic acid, a role that aldehyde, for example, could also fulfil. And he often expressed his astonishment that some biochemists kept on trying to isolate and identify the 'so-called co-zymase'.

Thus the first reason for Kluyver's abstention from enzyme research was that his own venture into the field had yielded such unpromising results. The second was that his philosophical approach had led him to the conclusion that enzymes involved in oxido-reductions could not be expected to exhibit the extreme specificity that current concepts ascribed to them. Instead, they should possess other properties, more pertinent to the problem of their activity, and he felt that these could be studied more effectively by methods not primarily aimed at their isolation.

In the third of the London lectures he developed his objections to the notion of rigorous specificity. Granting that the individual step reactions might be enzyme-controlled, he reasoned that this view should inevitably lead to the undesirable consequence that a distinct and separate enzyme had to be postulated as the necessary catalyst for each step. This would mean that such reactions could then be 'explained' by invoking named, though unknown catalytic entities; it would imply that 'a living cell should be considered as an arsenal filled up with enzymes which successively are brought into action', and that the distribution of such enzymes in various organisms would be entirely fortuitous and unpredictable. Finally, on account of:

the bewildering diversity of compounds which are able to act as dehydrogenation substrates for the cells of *Pseudomonas putida* (for which earlier the ability to grow

at the expense of some eighty different simple organic substances, belonging to several structurally unrelated groups, had been mentioned) it will be generally agreed that here the doctrine of extreme specificity becomes untenable. For it can scarcely be conceived that the cells of the bacterium in question contain as many dehydrogenases as there are suitable oxidation substrates for these cells. And, moreover, we should be obliged to assume that these cells have at their disposal specific catalysts for substrates such as bromo-succinic acid and bromo-propionic acid which do not occur in nature, and which are only made by the conscious operations of the organic chemist ((23), pp. 97–9).

As a satisfactory escape from such consequences he suggested that enzymes operative in oxidation-reduction reactions have multiple potentialities. After arguing that the indubitable specificity of hydrolases was conditioned by structural features, he stated that:

a catalyst can only be expected to be non-specific, i.e. be able to promote various reactions, in so far as these reactions are of the same nature. Now, we have seen that the primary reactions to which biochemistry can be reduced satisfy this demand in a high degree: all these reactions are either of the hydrolytic or of the oxido-reduction type. So at first sight it seems quite conceivable that the same enzyme can promote several different conversions... With reference to what physical chemistry has shown in regard to the mechanism of catalytic action, we must conclude that the first condition of catalysis is the formation of a loose compound between the catalyst and the substrate that is to be activated. So it will be clear that one condition which may determine the specificity of a catalyst may be its steric configuration. But a second condition which must be fulfilled is that the formation of the afore-mentioned compound should give rise to a suitable activation of one or more atoms of the substrate. Bonds between these atoms and the rest of the molecule must be weakened, and since nowadays the nature of the chemical bond is universally accepted as being of an electrostatic nature, the activation must be due to the polarising action of an electric field of the catalyst. But then the logical inference will be that specificity of a catalyst may also be due to the intensity of its electric field ((23), pp. 96–7).

This led to the concept that one enzyme, like a master key, would be able to activate a number of different substrates.

Once accepting the presence of such dehydrogenating 'master keys' in bacterial cells, there is no clear reason why one should not go farther and accept the supposition that in *Pseudomonas putida* there is only a single oxido-reduction promoting agent which acts on all the substrates mentioned... And the same conclusion holds good for all the primary oxido-reductions which together constitute the typical fermentation process of a cell.

This does not imply, however, that no specificity at all exists in oxido-reduction promoting agents. On the contrary, we shall have to seek, in the differences of the electrostatic properties of the agents of different specific cells, the explanation why some of these cells dehydrogenate sugars only, others hydrocarbons as well, still others methylamine or nitrites. And we may cherish the hope that the time will come when a well-founded quantitative theory of catalysis will lead to a sharp characterization of the electrostatic properties of the different catalysts... ((23), p. 99).

These quotations show that Kluyver had developed a concept as to how the general properties of living cells as oxido-reduction promoting agents might be better understood and studied. The search for increasingly comprehensive fundamentals thus took the direction of determinations of the redox potentials

in cultures of metabolizing cells, undertaken in the hope of thereby contributing to the quantitative evaluation of biochemical potentialities. The papers dealing with this phase of research (52-58) show again the judicious selection of organisms and substrates for such studies, as well as the awareness of methodological pitfalls and the ability to devise solutions for eliminating them. But this line of approach was not long pursued; the results, though suggestive, could not truly be interpreted as providing the desired information.

In later years Kluyver obviously had to revise his ideas about enzymological research. In a way, this was not too difficult because the discoveries of common pathways, of universally occurring cycles, and of a very small number of functional groups obviated the need for assuming an unlimited number of randomly distributed enzymes in living cells. The general participation of phosphopyridine and flavin nucleotides in substrate dehydrogenations strikingly supported his concept of a common principle for hydrogen activation, and the graded redox potentials of these co-enzymes and of the cytochromes are, of course, in line with the ideas expressed in the London lectures. Moreover, the phenomenon of induced enzyme synthesis implies that the enzymes required for the initial transformations of the numerous substrates that an organism can utilize are not inevitably a permanent feature of its enzymic composition, which made it easier to accept the high degree of specificity that modern enzyme research has revealed. How well versed Kluyver was in these new developments is evident from the Harvard lectures (24) in which the latest details of metabolic processes are presented.

By the time when more or less satisfactory methods had been developed for the preparation of enzyme extracts from bacteria, Kluyver was not in a position to take advantage of the situation. After the surrender of Holland to the Nazi forces the activities of the Delft laboratory soon came to a virtual standstill. Initially it was still possible to conduct studies on some aspects of applied microbiology, but later these, too, had to be abandoned or carried on clandestinely. In the latter category belongs the work on the manufacture of penicillin, carried out in collaboration with the Netherlands Yeast and Alcohol Manufactory. The success of this enterprise is attested to by the fact that soon after the end of the war this concern emerged as one of the leading penicillin producers, marketing a product of such purity that, according to reports, it elicited the admiration of Sir Alexander Fleming.

When, after five years of subjugation and pillage, Holland was liberated, conditions for the resumption of scientific work were extremely bad. And Kluyver, who had been mentally and physically worn out during the war years, did not experience the elation that characterized the response of so many others. There was, to be sure, a feeling of relief; but for several years he remained despondent. An important contributory factor to this attitude was the recognition that he had lost touch with the new developments in science. Through the initiative of many friends and admirers abroad he soon began to receive reprints, journals, and books, covering the advances made in the interim during which he had been shut off. But the very bulk of this literature frightened him; he despaired of ever being able to digest and comprehend the

newer developments to the point where he could once more start working in a manner that would not be outmoded. Those who knew his practically unlimited capacity for work, integration, and comprehension expected that, unless the war had inflicted permanent damage to his mind and physique, the chances for his return to the position of eminence he had so long held would be excellent. And in a short time he had indeed brought his knowledge up to date; this is clear from a perusal of his later contributions (24, 59, 60, 61).

But soon other duties were assigned to him which made it increasingly difficult to guide personally the work in his institute. He was elected President of the Koninklijke Nederlandse Akademie van Wetenschappen, a position from which he resigned in 1954; he served as Rector Magnificus of the Technological University; he was appointed to numerous committees. Owing to his strong sense of responsibility all these functions implied that he spent much of his time on matters not directly concerned with the development of microbiology and biochemistry. Although this may be deemed regrettable by workers in these fields, it certainly was of great benefit to the institutions and committees on which he served, where his opinions, always based on an exhaustive study of the problems and deep concern for the well-being of his fellow-men, carried much weight. Professor Bottema, Rector Magnificus of the Technological University at the time of Kluyver's death, referred to this aspect in his interment speech: 'I speak also on behalf of the Senate, simply in order to say that from its midst has been taken he who was by far the first. In our memory he stands in his accustomed place in the Senate room, speaking in his characteristic attitude, advising and warning and offering a solution; and we knew that it was right to accept his suggestions and to follow the road he indicated.' Many are they who have been privileged to experience the deep and humane wisdom of Kluyver's advice, and the ever gentle manner in which it was given; they know that this moving tribute is no exaggeration.

And still other matters laid claim to his time. Perhaps most important among them were the invitations he received to participate in scientific meetings. For the first few years after the end of the war he abstained from such activities, feeling that he could not contribute anything of value. For the same reason he denied himself a number of opportunities to travel abroad and so acquaint himself more directly with developments about which he had learned only through a study of the literature. He did not wish to profit in this manner without being able to reciprocate, and he was too modest to accept the argument that the new pioneers would benefit at least equally from his vast experience and critical ability. But not for long could he maintain this attitude; in connexion with his official positions, and as a result of many honours that were bestowed upon him,* he found himself more and more often

* He was the recipient of several honorary doctorates (Iowa State College, University of Louvain, Rutgers University, Swiss Federal Institute of Technology); he was elected to many foreign scientific societies and academies (Hon. Mem. Soc. Amer. Bacteriologists, and Soc. Gen. Microbiology; For. Mem. Finnish, Royal Flemish, and New York Acad. Sci.; For. Mem. Royal Soc.; For. Hon. Mem. Amer. Acad. Arts and Sci.; For. Assoc. Nat. Acad. Sci. U.S.A.); in Denmark he was awarded the Emil Christian Hansen Medal, and in England the Copley Medal of the Royal Society.

in a situation that unavoidably called for the delivery of special lectures ((24), (59), (66)). The enormous amount of effort spent in preparing such lectures, in planning different ways for fitting the pieces of a selected topic together into a coherent story, in digging up details that might be useful in developing or illustrating an argument, in searching for the most felicitous phrases in which the ideas could be formulated, is difficult to imagine except for those who know from experience how the master worked. The approach was the same in the preparation of publications; his collaborators became familiar with the general prescription: you must assume that the reader is unfamiliar with the subject; therefore you must introduce it in a simple manner; and then you must take the reader by the hand, and by logical, small steps guide him to the point where the conclusions you have reached become inescapable. They also learned that, even after a manuscript had been revised six or eight times, his logical and critical mind would still detect previously unnoticed flaws in certain passages which, at the expense of hours of additional argument and work, would be eliminated by rearrangement or rephrasing. This perfectionist attitude instilled into his pupils an appreciation of the value of paying attention to detail. But it also implied that Kluyver had to devote a great deal of time to the preparation of a lecture or manuscript before he had accomplished an effect that finally satisfied him.

Then, too, there was the new institute that required constant attention. Starting in the mid 'twenties the activities in his laboratory had increased. The formal lecture and laboratory courses, attended by a very few students during the first years, had begun to draw increasingly larger numbers, as not only graduate students from the chemistry division of the Technological University, but also biology majors from other Dutch universities, especially from Leiden and Utrecht, had come to realize that the opportunity for acquiring a sound and general background in microbiology and biochemistry was unequalled elsewhere. The fascination of the new, rapidly developing subject made many of them decide to prepare a doctor's dissertation under Kluyver's direction. And in the middle 'thirties had begun the influx of more advanced foreign workers who were desirous to extend their training at what had become the unchallenged world centre of microbiological research. These came from America, Denmark, Sweden, Belgium, France, Israel, South Africa; they returned to their countries with a wealth of new information and ideas, and with indelible memories of the noble personality who had created and maintained the stimulating and generous atmosphere characteristic of his institute. Hence the space and facilities of the laboratory that had been built for Beijerinck, and used with only minor alterations and additions since 1911, when a new wing had been added, were often severely taxed. The situation became even worse when new areas of research, such as the unit for electron microscopy, were added.

The vast expansion programme of the Technological University after the war therefore included the eventual construction of a new microbiological institute. The planning naturally involved Kluyver in endless conferences concerning major decisions as well as detail. He strongly and successfully

argued in favour of a laboratory with attached living quarters for the director, knowing from experience how greatly would thereby be facilitated the maintenance of a close contact with the work.

However much time Kluyver devoted to this project, he knew that it was spent on behalf of his successor, who would thus be provided with a home and laboratory designed as carefully as possible. The new institute was scheduled to be ready for occupation in 1957. Kluyver was to retire only a year later. The prospect of retiring he dreaded. For 35 years he had guided and inspired the work of his associates and students; he had dedicated his life to science, and not permitted himself the time to develop outside interests. It was difficult to imagine how the void following upon his 70th birthday would be filled.

In his inaugural address he had expressed his expectations with regard to the fate of Beijerinck's institute under the new direction. With his usual and genuine modesty he had addressed to his famous predecessor the remark: 'It would be fruitless for me to attempt to maintain the lustre that your institute has acquired by virtue of your work. But to contribute to making this golden quarter century of Dutch microbiology something that will continue to live in the minds of a new generation, that is within my power.' There can be no question that the career of Albert Jan Kluyver as a microbiologist has not in the least diminished the lustre of Beijerinck's institute; in fact, it is apparent that the Delft laboratory of microbiology really came into its full glory during Kluyver's tenure of the chair.

This was not the result of a preoccupation with technological applications of microbes, so strongly emphasized in the inaugural address. These were not neglected. But the importance of Kluyver's work stems from his philosophical outlook, which made him realize the great importance of micro-organisms as a means of penetrating more deeply into the fundamental problems of life. This aspect has been beautifully developed in the lecture he delivered in 1955 before the combined sections of the Koninklijke Nederlandse Akademie van Wetenschappen (65), a lecture he used to refer to as his 'swan song'.

Notwithstanding the universal recognition of Kluyver's noble personality and scientific eminence, the last 15 years of his life had not been happy ones. The war had left wounds that never quite healed; his health was precarious; and the death, in 1952, of his wife, the deeply understanding and devoted companion and friend for 36 years, was a blow to which he resigned himself with great difficulty. He had lost the glowing enthusiasm for his work that had characterized the early years, and the old slogans had been replaced by new ones, such as 'This is a great and terrible world', more pessimistic, more in keeping with his fears for the future of humanity. He took little interest in his health; though suffering from angina pectoris, he stuck to his strenuous schedule of work for seven days a week, from 9 a.m. till midnight or after, with at most a token vacation of a few days a year; he continued to drink large quantities of strong coffee and to smoke incessantly, although he made the concession of restricting himself to one cigar per day, and often to smoke cigarettes with a low nicotine content to which he was wont to refer as 'innocence'. No doubt he realized that this regime might shorten his life;

but he did not seem to care any longer. Only his most intimate friends were aware of his rapidly declining health; never would he burden others with such knowledge. They might, it is true, notice the signs of extreme fatigue at the end of the day. But Kluyver had a phenomenal capacity for recovery and after a night's sleep could put up a front of being in good condition. As usual, he was at work in his study after midnight on Sunday, 13 May. That night he died of a heart attack....

Thus came to an end the life of a man who, by his example, has erected a monument to the power of intelligence, guided by great compassion, and coupled with undeviating dedication to his work; and who in consequence has engendered more inspiration and devotion than falls to the lot of most people in a comparable situation.

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Bacteriocinogenesis in *Bacillus megaterium*

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SUMMARY: A strain of *Bacillus megaterium* has been found under certain conditions to liberate a bacteriocin-like agent which has been termed 'megacin'. The conditions necessary for induction of megacin formation in this strain are reminiscent of those leading to induction of bacteriophage in other (lysogenic) strains of the same species. Megacin, however, differs from bacteriophage in not giving rise to any further production of antibacterial principle, after acting on bacterial cells. Induction of megacin formation by ultraviolet irradiation is dependent on the composition of the medium. Only in a complete medium is megacin produced, although certain preliminary stages in the induction process and a concomitant, reversible photobiological effect can take place in minimal nutrient medium.

We have reported recently (Ivánovics & Alföldi, 1954, 1955) that within *Bacillus megaterium* species mutual antagonism between strains is a frequent phenomenon which can be attributed to a water-soluble antibacterial substance of protein character designated by us as megacin. In some strains megacin production is greatly enhanced by small doses of ultraviolet (u.v.) light. After growth for 90-120 min. mass lysis ensues with liberation of megacin into the medium. On account of its mode of formation and its properties we classed megacin with the bacteriocins, yet it differs essentially from the known bacteriocins, e.g. colicin and pyocin (Fredericq, 1953; Jacob, Siminovitch & Wollman, 1952; Jacob, 1954) in that it acts on all strains of its own species and not only on some individual 'sensitive' ones. Nevertheless, the antibacterial spectrum of megacin is very narrow; besides certain strains of *Bacillus subtilis* and *B. anthracis*, megacin acts only on the pigment-forming non-pathogenic micrococci, e.g. *Micrococcus aurantiacus*, *M. cinnabareus* (Ivánovics, Alföldi & Ábrahám, 1955).

Lysogenesis, as studied extensively in *Bacillus megaterium* (Lwoff, 1953*a*), and megacin formation are strikingly analogous in several ways. In order to determine whether these two phenomena are physiologically related, the conditions necessary for induction of megacin formation by *B. megaterium*, strain 216, were studied in greater detail.

MATERIALS AND METHODS

The experimental techniques used were the same as those described in our recent publication (Ivánovics & Alföldi, 1955).

Megacin-forming strain. A *Bacillus megaterium* strain isolated, and designated as no. 216, by us (Ivánovics & Alföldi, 1955) was used in these studies.

Complete nutrient media. These were prepared as described earlier (Ivánovics & Alföldi, 1955), namely yeast extract and acid-hydrolysed casein (yc), yeast

extract peptone medium (YP), and yeast extract plus enzymically digested casein (YDC) medium. This latter medium was earlier designated by us as medium-9. The agar media were prepared by adding 15 g. of agar to each litre of the liquid media mentioned above.

Defined medium. A lactate ammonium chloride nutrient (Friedlein, 1928) was used as basal medium. *Bacillus megaterium* strain 216 develops extremely slowly in this medium alone; yet it makes a useful medium when supplemented with 1 % glucose and 0.05 % ammonium glutamate (FGG medium). Glucose was sterilized separately by filtration and added to the nutrient immediately before use.

Cultivation, and induction with u.v. light, of strain 216. The organisms were grown at 35° in 100 ml. Erlenmeyer flasks containing 20 ml. of the nutrient solution, with gentle shaking. The inoculum consisted of 0.2 ml. of bacterial suspension of optical density 0.40 prepared from a 24 hr. YDC or FGG agar culture of the strain. The optical densities of bacterial suspensions and cultures were measured with an 'Oriphot' (Elektromos Mérő-eszközök Gyára, Budapest) microphotometer with a photoelectric vacuum cell. Measurements were carried out in test tubes 14.5–15.0 mm. in diam., using an orange colour filter (610 m μ .).

The source of the u.v. light was a 'Hanau' high-pressure mercury lamp used at a distance of 25 cm. under the conditions described earlier (Ivánovics & Alföldi, 1955). The energy emitted by the lamp at 25 cm. distance in the optical axis amounted to 7.3×10^5 erg./cm.², of which 3.3×10^5 erg. fell to the spectral range below 3500 Å. The bacterial suspensions were poured in Petri dishes in a layer not more than 2–3 mm. thick and irradiated with u.v. light while the vessel was gently swirled by hand.

Count of colony-formers. Samples (0.1 ml.) of an appropriate dilution of culture were plated on the surface of the agar medium.

Bacterial cell count. The cultures were diluted with aqueous methyl violet solution (0.02 %, w/v), and the numbers of cells and chains were counted in a Buerker haemocytometer.

Megacin titration. A highly sensitive variant of the 'mutilate' strain of *Bacillus megaterium* (Pasteur Institute, Paris) was used as the indicator strain. One ml. of a suspension of the strain, optical density 0.20, was mixed with an equal amount of melted YP agar and layered upon a plate 10 cm. diam. prepared from the same agar. Serial dilutions of the lysate were then dropped on the plates, and the megacin titres determined. The highest dilution of lysate capable of giving a partial inhibition of growth varied from 1:10,000 to 1:40,000 in individual experiments carried out under different conditions. The megacin content of the lysate in a control culture grown in YDC medium and induced with u.v. radiation under optimal conditions was taken to be 100 % and all other values referred to it.

Stained preparations. Bacteria on slides were kept for 10 min. in Bouin's fixing solution, washed, treated with tannic acid, and stained with methyl violet (10 sec., 1 %, w/v, solution) according to Gutstein (1926).

RESULTS

Induction in complete medium

Growth of induced bacteria. Young, 3–4 hr., cultures were exposed to u.v. radiation for 30 sec. An example of the behaviour of cultures after induction with u.v. radiation is represented in Fig. 1. At about 90 min. after irradiation, the culture began to lyse. The antibacterial titre of the lysate was found to be 1:25,000.

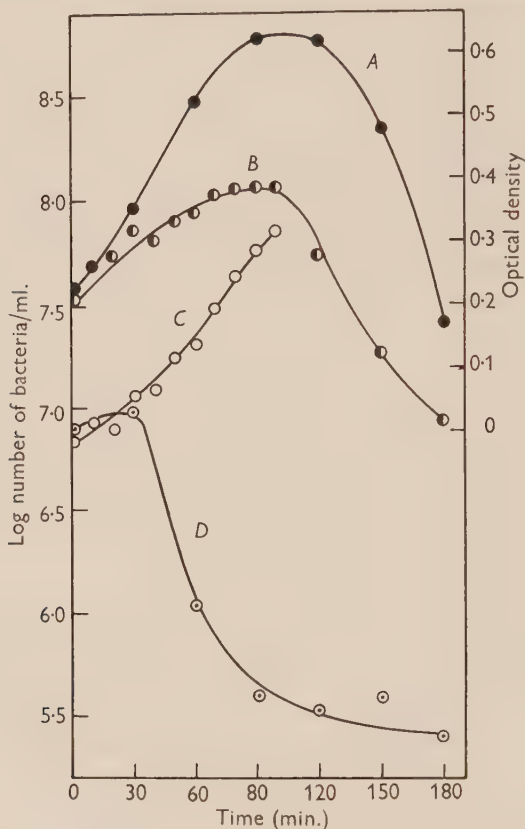


Fig. 1. Growth after u.v. irradiation of culture of *Bacillus megaterium*, strain 216, grown in YDC medium. In this and all other diagrams the time of irradiation is zero time. Irradiation for 30 sec.; A, optical density; B, bacterial count; C, chain count under the microscope; D, number of colony-formers on YDC agar.

In this and similar experiments it was observed that the bacteria gradually increased in number until lysis occurred. Nevertheless, bacteria plated out on the surface on nutrient agar in their early phase of growth after irradiation were still capable of forming colonies.

These experiments indicate that the lysis of irradiated bacteria does not begin until after the first generation following irradiation.

The morphology of induced cells. The finer cytologic structures, e.g. the nuclear structure, have been disregarded in our investigations, cell integrity alone being considered at this stage. Cell size and the condition of cell walls

in stained or unstained, living preparations, were examined with the phase-contrast microscope.

In young cultures of our strain, as in *Bacillus megaterium* strains in general, the septa are formed before the cells are fully developed in size. Thus the bicellular form is typical (Pl. 1, fig. 1). Not infrequently, the cells are linked by cell-wall remnants to form slack chains. After induction, the septate cells soon decrease in number and, correspondingly, the individual cells increase in size; they become more and more elongated, yet division does not cease, as is clearly indicated by the rising number of cells estimated by direct count (Fig. 1, curve *B*). On comparing the morphological details with the number of cells in induced cultures it appears obvious that the rise in density during growth after irradiation is made up of two components: elongation and division of the cells. After 60 min. some individual cells no longer stain evenly, so that the cell wall is not everywhere visible (Pl. 1, fig. 2). The cells become more and more varied in their morphology, and the proportion of abnormal cells increases. Besides faintly staining cellular remnants, a few cells more or less disintegrated are found (Pl. 1, figs. 3-5), but perfectly normal forms still occur.

These observations do not necessarily mean that the lesions in induced cells are primarily lesions of the cell walls. Under the phase-contrast microscope, vacuoles are seen in the cytoplasm in steadily increasing numbers, and it appears rather that bacterial disintegration is preceded by increased permeability of the cell wall. Support for this view is afforded by the observation that on applying Gutstein's (1926) staining method the tannic acid, used as a mordanting agent, penetrates the cytoplasm allowing the cell to be uniformly stained (see Pl. 1, figs. 2, 3).

Re-incubation, in a new medium, of cells irradiated in YDC nutrient. After u.v. irradiation, cultures grown for various lengths of time were cooled rapidly and centrifuged between 8 and 12°. Thereafter, the bacteria were suspended in fresh nutrient solution to allow growth to continue. If the sediment was resuspended and re-incubated in complete medium such as YDC, YC, YP, or horse meat-extract peptone broth, after a definite period of growth the culture underwent mass lysis with production of megacin. Lysis was not inhibited even when the transfers of cells into fresh media were made immediately after the irradiation of the culture. Thus under these circumstances there was no evidence for reversibility of the induced state, such as was found when irradiated cells were transferred to the surface of a solid medium.

It was quite otherwise when irradiated cells were transferred, after the lapse of different periods of time, to the defined FGG nutrient medium (Fig. 2). For 45 min. the change in the culture treated with u.v. radiation was completely reversible. After longer periods of time the transferred cells were found to be already disintegrated, yet in the lysate there were only small amounts of megacin. This suggests that lysis of cells and megacin production are not strictly parallel processes. Synthesis of active megacin is apparently not complete until the very end of the residual growth period, and is contingent upon adequate conditions in the medium.

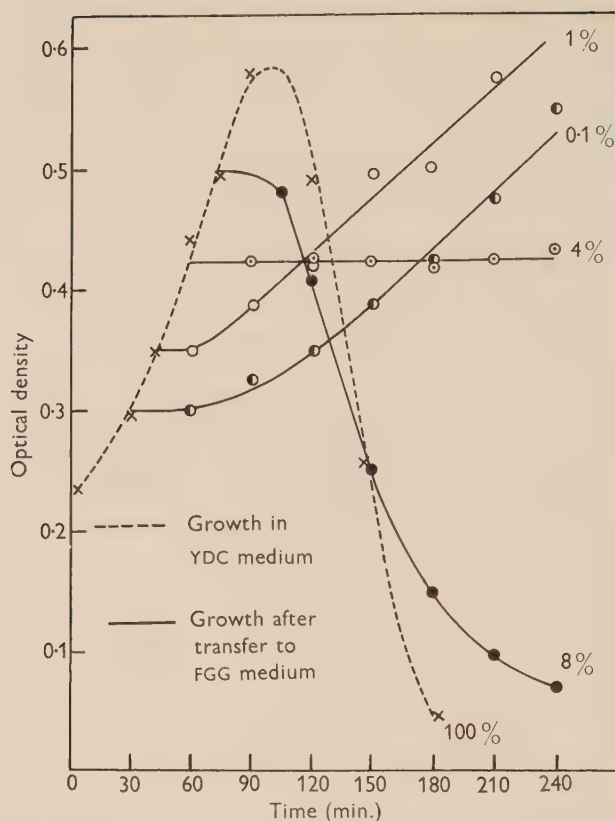


Fig. 2. Behaviour of bacteria, grown in YDC medium and irradiated with u.v. light, after their transfer to FGG medium. *Bacillus megaterium*, strain 216, grown in YDC, irradiated for 30 sec., and reincubated. 10 ml. samples taken at various times thereafter. The bacteria were washed and reincubated in FGG medium. The figures by the curves indicate the amounts of megacin produced (% of max.).

Induction in FGG (defined) nutrient medium

Characteristics of irradiated cultures. In FGG medium seeded with the standard inoculum (grown on FGG agar), maximal growth varied from 0.50 to 1.00 optical density in different experiments.

Young cultures exposed to u.v. radiation (10 sec.) had usually not lysed after 24 hr. of reincubation. Occasionally, however, a very slight reduction in density was observed within 3 hr. When this occurred a small amount of megacin, less than one-tenth of the amount produced under optimal conditions in YDC medium, was found in the culture. In other cases, when practically no reduction in optical density was observed, only traces of megacin were formed in FGG medium.

Although in the defined medium u.v. irradiation was followed neither by appreciable lysis nor by megacin production, some photobiological effect was nevertheless apparent when the cells were plated on to the surface of an agar medium. As can be seen in Fig. 3 the optical density of the culture

grown in FGG medium and irradiated, reached a maximum at about 200 min. and thereafter remained constant. Cell division ceased about 90 min. after irradiation, which means that the further rise in optical density was due solely to elongation of the cells. It was notable that when the irradiated bacteria

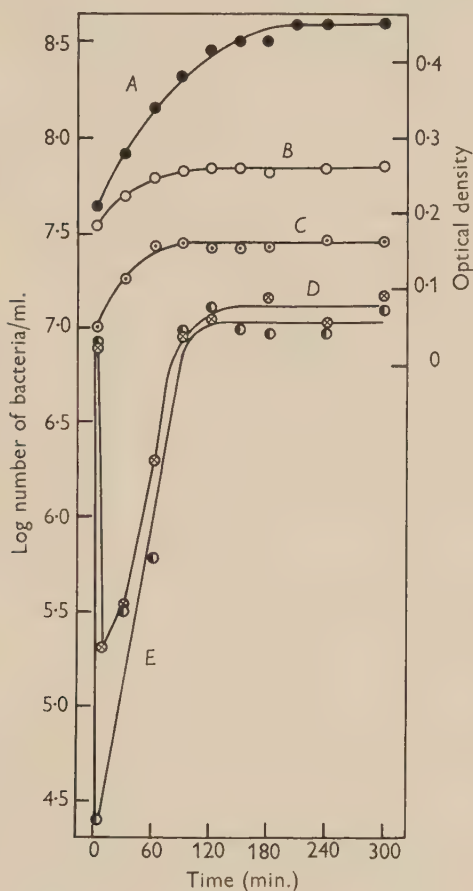


Fig. 3. Young culture of *Bacillus megaterium*, strain 216, grown in FGG medium, irradiated and allowed to continue growing. A, optical density; B, total bacterial count estimated in the haemocytometer; C, individual chain count; D, E, number of colony formers obtained on FGG and on YDC agar, respectively.

were plated immediately on to nutrient agar, only 0.3 % of the colony-formers originally present had yielded colonies in YDC medium, as compared with 2.4 % in FGG agar. However, this effect gradually disappeared; when plated out 90 min. later the colony counts slightly exceeded their initial values.

Subculture in YDC medium of bacteria irradiated in FGG medium. The experiment shown in Fig. 4 demonstrates that both lysis and megacin production ensued when cells cultured and irradiated in FGG solution were transferred to complete yeast extract plus casein digest medium. The figure shows the effect of transfer at times up to only 90 min. after irradiation, but closely similar

results were observed when bacteria, irradiated in FGG medium, were transferred to complete nutrient solution as long as 5 hr. after the u.v. treatment. Thus, although in the defined medium irradiated bacteria ceased multiplying and growing after 200 min., they retained their 'induced' state several hours.

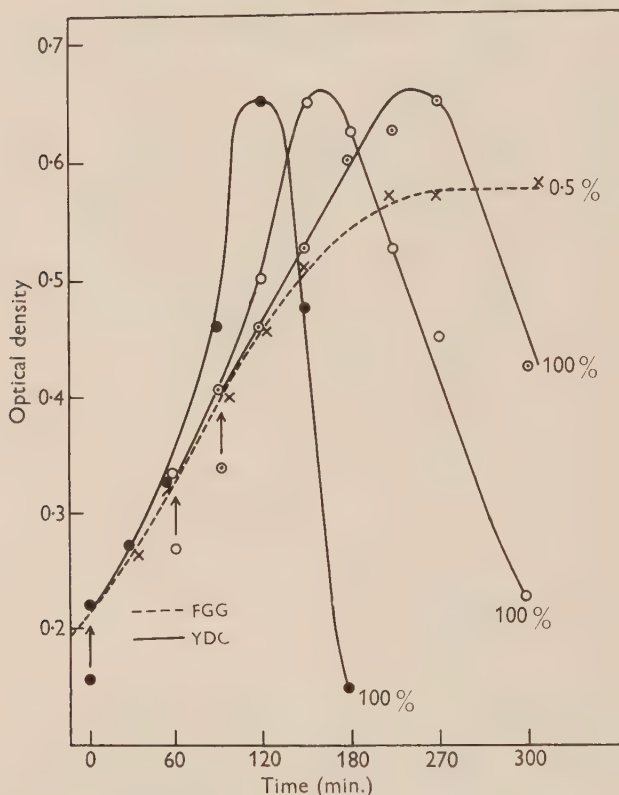


Fig. 4. Behaviour of cells induced in FGG medium after their transfer to YDC medium. Young culture of *Bacillus megaterium*, strain 216, grown in FGG medium, irradiated for 10 sec. and resuspended in 10 ml. of YDC medium and reincubated. The figures on the curves indicate the amount of megacin produced as percentage of maximum. Arrows show transfer, after irradiation, from FGG to YDC medium.

Morphology of the induced cells. The changes observed in bacteria irradiated and subcultured in defined medium can be summarized as follows. During the first 90 min. the individual rods did not differ in outward appearance before and after irradiation; after 90 min. irradiated cells gradually became elongated, but not even in preparations made 260 min. later was any disintegration or lysis observed. This morphological picture corresponds with findings represented in Fig. 3.

DISCUSSION

As we have already described (Ivánovics & Alföldi, 1955) some of the *Bacillus megaterium* strains are bacteriocinogenic. This fact opens up new possibilities for the study of what is called lethal biosynthesis. Some such individual

strains lyse gradually when irradiated with u.v. light and subcultured. This is associated with the accumulation in the lysate of a considerable quantity of the bacteriocin designated by us as megacin (Ivánovics & Alföldi, 1954). Our present experiments show that after u.v. treatment 97–99% of the cells of our inducible *B. megaterium* strain lyse. This is about the same proportion as that observed in the lysogenic strains of this species.

So far we have failed to demonstrate any lysogenic property in *Bacillus megaterium*, strain 216. In the lysate obtained after induction we were unable to detect even a single infective phage particle, although lysate corresponding to 10^8 cells had been plated out on various indicator strains of *B. megaterium* sensitive to temperate phages. Furthermore, no phage particles were seen in lysates of strain 216 under the electron-microscope (Ivánovics, Alföldi & Lovas, to be published). No infective phages were found either in lysates obtained after u.v. irradiation or in any cultures of our megacinogenic strain grown on liquid or solid media. We think it essential to stress this point, because the behaviour of our strain following irradiation with u.v. light is in many respects reminiscent of the behaviour of the typically lysogenic strain 899 (1) of *B. megaterium* (Lwoff, Siminovitch & Kjeldgaard, 1950; Lwoff, 1953*b*). But while after induction this lysogenic strain 899 (1) produces phage, strain 216 produces megacin. Our strain also differs markedly from the strain 91 (1) studied by Lwoff & Siminovitch (1952) which lyses after irradiation but whose lysate contains neither phage nor bacteriocin.

The experiments undertaken so far do not enable us to answer the question whether megacin is a non-reproductive product of some prophage which may be carried by strain 216 and may govern its hereditary character. The similarities observed between the lysogenic *Bacillus megaterium*, strain 899 (1), and the bacteriocinogenic strain 216 in their induction, support this hypothesis. On the induction of lysogenic *B. megaterium*, strain 899 (1), by u.v. light Lwoff *et al.* (1950), as also Delaporte & Siminovitch (1952), observed first division, then elongation, and finally lysis of cells occurring in the second generation following irradiation. The cells of our bacteriocinogenic strain divide for half an hour after irradiation without any essential changes in shape or size. In the second generation following irradiation the cells have already become elongated, although cell division still occurs. The cells are twice their original size, but at the same time gradual disintegration begins. In the first generation the process of induction is still reversible if the cells are plated on the surface of nutrient agar. In inducible lysogenic bacteria this is a well-known phenomenon (Lwoff, 1953*b*). With our strain the process is also reversible when the irradiated cells are transferred to a defined medium.

On the evidence presented it would appear that lysogeny and megacinogeny are somehow interconnected, although we do not yet know how.

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EXPLANATION OF PLATE

Culture of *Bacillus megaterium*, strain 216, grown on YDC medium, irradiated for 30 sec. and re-incubated. Stained preparations made at various times. Fig. 1, before irradiation; 2, 60 min. after; 3, 90 min. after; 4, 120 min. after; 5, 150 min. after irradiation. Cell-wall staining after Gutstein. $\times 1800$.

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G. IVÁNOVICS & L. ALFÖLDI—BACTERIOCINOGENESIS IN *B. MEGATERIUM*. PLATE 1

(Facing p. 530)

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The Effect of some Amino Acids on the Growth of Two Varieties of *Ophiobolus graminis*

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SUMMARY: An examination was made of the effect of certain amino acids on the growth of three isolates of *Ophiobolus graminis* Sacc. and of six isolates of *O. graminis* var. *avenae*, either as sole sources of nitrogen, or as supplements to ammonium nitrate. In all cases growth with aspartic acid was equal or nearly equal to that obtained with hydrolysed casein. Lysine and threonine were strongly inhibitory. The two varieties differ from each other in their reaction to cystine and cysteine, *O. graminis* itself making as good growth with these amino acids as with hydrolysed casein, and var. *avenae* being inhibited in growth.

Ophiobolus graminis Sacc. var. *avenae* differs from *O. graminis* itself in pathogenicity, being capable of causing a root rot (take-all) in oats as well as in wheat, in ascospore length, and in its capacity to make good growth in expressed sap from oat roots which does not support growth of *O. graminis*. The two varieties resemble each other closely in general morphology, in the type of infection and development of the disease in wheat, and in sensitivity to an inhibitor of growth and respiration obtained from oat sap (Turner, 1956). An investigation into the effect of amino acids on growth of the two varieties has been made. Here also, while resembling each other in certain well-marked reactions, in particular in intolerance of lysine and threonine, the two varieties can be distinguished from each other in their response to the sulphur-containing amino acids cystine and cysteine.

METHODS

Fungal isolates. The isolates previously described (Turner, 1956) were used. They are designated as W or O according to spore length, W isolates belonging to *Ophiobolus graminis*, O isolates to *O. graminis* var. *avenae*. All W isolates grew well in a defined medium, as did isolates O1, O2, O3, and O4. The remaining O isolates grew poorly. All were mycelial isolates from infected roots of wheat or oats.

Measurement of growth. The isolates were grown in a defined medium of the following composition per l.: 1.0 g. KH_2PO_4 ; 2.0 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 3.0 g. Na_2HPO_4 ; 20.0 g. glucose; 1 mg. biotin; 1 mg. thiamine. This will be referred to as basal medium. To this was generally added 5.0 g. ammonium nitrate/l., the medium then being called 'AN basal'. Additions of hydrolysed casein or amino acids were made as described. These gave the same results whether autoclaved (120° for 10 min.) or sterilized by filtration through sintered glass

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and were therefore added before autoclaving. Glucose was autoclaved separately and added aseptically to the flasks. Flasks of 100 ml. capacity were used, each containing 5 ml. medium; four replicates were set up for each treatment. Each flask was inoculated with one disk 3 mm. in diam. cut with a drawn-out glass tube from the growing edge of mycelial mats on 2% malt agar. The flasks were incubated at 25° for 5½ days and the mats were then dried, washed and weighed. A control was run for each experiment with AN basal medium alone; the results are generally expressed relative to growth on this medium. In most cases each figure represents the average result of three separate experiments.

RESULTS

Survey of amino acid effects. The addition of 0.05% (w/v) hydrolysed casein (Oxoid) to the AN basal medium increased growth of *Ophiobolus graminis* isolate W₁ by about 40% over the control. In the absence of an inorganic nitrogen source a mixture of 19 amino acids and asparagine and glutamine, each at a final concentration of 0.0025M (giving a total addition by weight of 0.55% (w/v)) when compared with 0.55% (w/v) hydrolysed casein gave equal growth.

Table 1. *Effects on growth of isolates of Ophiobolus graminis (W) and O. graminis var. avenae (O) of amino acids added singly (0.004M) to AN basal medium*

For precise definition of classes see text. Isolates O₂, O₅, and O₆ tested only with aspartic acid, glycine, threonine, lysine, methionine, cystine and cysteine.

A. Good for all isolates (classes 1 or 2):

L-Asparagine, L-aspartic acid

B. Good to ineffective for all isolates (classes 1, 2 or 3):

L-Glutamine, L-glutamic acid, L-proline, DL-valine

C. Ineffective or poor for all isolates (classes 3 or 4):

L-Leucine, DL-isoleucine, DL-phenylalanine

D. Poor or bad for all isolates (classes 4 or 5):

DL-Threonine, L-lysine, DL-methionine

E. Good for W isolates, poor or bad for O isolates:

Class ...	1			2		4		5				
L-Cystine	W ₁	W ₃	W ₅	—		O ₂	O ₆	O ₁	O ₃	O ₄	O ₅	
L-Cysteine	W ₃			W ₁	W ₅	O ₂	O ₆	O ₁	O ₃	O ₄	O ₅	

F. Effects variable:

Class ...	1	2			3	4				5
Glycine	—	W ₁	W ₃	W ₅	O ₄	O ₁	O ₂	O ₃	O ₆	O ₅
L-Alanine	W ₃	W ₁	W ₅		O ₁	O ₄	O ₅	O ₃		—
L-Serine	—	W ₃			W ₁	W ₅		O ₁	O ₃	O ₄
L-Histidine	—	W ₅	O ₃		W ₁	W ₃	O ₄	—		O ₁
L-Arginine	—	W ₅	O ₃		W ₁	W ₃	O ₄	—		O ₁
L-Tryptophane	W ₅	O ₃	W ₃		O ₄			W ₁	O ₁	O ₂
L-Tyrosine	O ₃	W ₅	O ₄		W ₃			W ₁	O ₁	—

Individual amino acids as sole nitrogen source were added singly to 0.04M and tested with isolates W₁ and O₃; as supplements to the inorganic source they were added to 0.004M (representing an addition of 0.053% for aspartic acid) and tested with six isolates, three of each variety; partial tests were made with three further isolates.

Growth in the control medium differed considerably between different isolates, as did growth with 0.05 % (w/v) hydrolysed casein. Comparison between isolates was therefore most easily obtained by broadly grouping results into five classes, viz. (1) good, growth about equal to that with hydrolysed casein; (2) moderately good, growth intermediate between that with hydrolysed casein and that in the AN basal medium alone; (3) ineffective, growth the same as in the control; (4) poor, some decrease in growth compared to the control; and (5) bad, growth less than 50 % of the control. Results given in Table 1 show (a) that asparagine and aspartic acid were as favourable to growth of all isolates as was hydrolysed casein, (b) that lysine, threonine and methionine were inhibitory to growth of all isolates, (c) that in general the O isolates were the more susceptible to growth inhibition by single amino acids, and (d) that, in particular, all O isolates were strongly inhibited, whereas the W isolates were stimulated in growth by cystine and cysteine.

When amino acids were added singly as sole nitrogen source to 0.04 M (only isolates W₁ and O₃ tested) the effects were similar except that leucine, isoleucine, valine, proline, tryptophane, tyrosine and phenylalanine were all poor nitrogen sources for both isolates (classes 4 or 5).

In their reactions to amino acids present singly in the medium the two varieties of *Ophiobolus graminis* do not differ much from the generalized scheme drawn up by Pelletier & Keitt (1954) from reports by different investigators on a score of fungi from all taxonomic groups. The clear distinction between the two varieties in their reaction to cystine and cysteine (in Pelletier & Keitt's grouping 'poor') are therefore of significance, at least indicating a difference in metabolism between them, and possibly being connected with their difference in pathogenicity.

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The Metabolic Significance of the Citric Acid Cycle in the Growth of the Fungus *Zygorrhynchus moelleri*

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SUMMARY: Cultures of the fungus *Zygorrhynchus moelleri* were grown in a glucose-acetate + ammonia + salts medium in the presence of either radioactive carbon dioxide, or acetate labelled in either the methyl- or the carboxyl-positions. After growth the protein was hydrolysed and the pattern of incorporation of radiocarbon into the amino acids was determined by chromatography and radioautography; activity from carbon dioxide appeared in glutamic and aspartic acids, methionine, threonine, isoleucine, proline and arginine. With labelled acetate lysine and leucine were also radioactive. A pool of free amino acids was present in this fungus.

Several amino acids were isolated from the three samples of hydrolysed protein and their specific activities were determined. Assuming that the tricarboxylic acid cycle operated in this organism, a number of predictions concerning the distribution of radiocarbon in certain amino acids which could be made were largely confirmed by the experimental findings. By partial degradation of glutamic and aspartic acids, followed by assay of the radioactivity in the products, it was possible to calculate that about 60 % of the oxalacetate used for citrate synthesis was recycled C₄-dicarboxylic acid; 40 % was synthesized from pyruvate and carbon dioxide. Approximately 25 % of the total respiratory carbon dioxide evolved could be accounted for in terms of decarboxylations occurring within the citric acid cycle.

A great deal of effort in recent years has been directed to assessing the importance in a number of micro-organisms of the tricarboxylic acid cycle as an operative physiological mechanism. The investigations may be divided roughly into two types: examination of the organisms for the presence of the requisite enzymes necessary to carry out the constituent reactions, and the use of isotopic tracer techniques to estimate the dynamics of the cycle as a whole. Investigations of the former type, while providing valuable information on the enzymic constitution of the cells, can offer no more than presumptive evidence for the working of the cycle as a unified entity.

In the latter group of investigations, organisms have been supplied with various compounds, often glucose, acetate, or carbon dioxide, labelled with radioactive carbon in one or more of their atoms. Suspected intermediates were isolated from the cells after a period of utilization of the labelled substrate and degraded to find whether the distribution of labelled carbon in the molecules corresponded with the predictions which could be made assuming that the cycle did operate. Thus Saz & Krampitz (1954), and Swim & Krampitz (1954*a, b*) showed in large-scale experiments with *Micrococcus lysodeikticus* and *Escherichia coli* that the carbon atoms of acetate labelled in either the carboxyl- or the methyl-positions equilibrated with the corresponding atoms

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of citrate, α -ketoglutarate, succinate, fumarate, and malate in a manner entirely consistent with the operation of a tricarboxylic acid cycle, but inconsistent with a dicarboxylic acid cycle.

Roberts, Abelson, Cowie, Bolton & Britten (1955), in a very extensive investigation of *Escherichia coli* growing on labelled acetate, carbon dioxide, etc., used the distribution of radioactive carbon in the amino acids of the protein as a means of estimating the magnitude of the flow of substances in various branches of the cycle. It was possible for them to determine the extent to which decarboxylations in the cycle were responsible for the production of respiratory carbon dioxide. Their methods have the advantage of requiring the isolation of amino acids rather than of minute quantities of transient intermediates, equilibration between glutamic acid, aspartic acid, and alanine, and their respective keto acids being assumed.

In the present investigation this technique is applied to a study of the flow rates in the Krebs cycle during the growth of the fungus *Zygorrhynchus moelleri*. It has already been demonstrated that this mould possesses all the enzymes necessary to carry out the sequence of reactions concerned in the tricarboxylic acid cycle (Moses, 1955), and it is now shown that the cycle plays a large part in the mechanism of terminal oxidations.

METHODS

The organism was grown in media containing either $^{14}\text{CO}_2$, or methyl- or carboxyl-labelled acetate as sources of ^{14}C . Several amino acids were subsequently isolated and their specific radioactivities determined. In addition, the intramolecular distributions of ^{14}C in the glutamic and aspartic acid specimens isolated were also measured. Determinations were made of the quantities of certain amino acids synthesized during the growth period, as well as of the increases in dry weight and the rates of respiratory gas exchange. The free amino acids present in the cells were investigated. The methods used are described in detail below.

Growth of the cells. *Zygorrhynchus moelleri* was grown in a glucose + NH_4Cl + salts medium (250 ml.) as in previous work (Moses, 1954). After growth for 13 hr. at 25° , the mycelium then being in its most rapid growth phase, the cells were centrifuged, washed, with a modified growth medium consisting of: $\text{NH}_4\text{H}_2\text{PO}_4$, 3.44 g.; KH_2PO_4 , 2.18 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g.; NaCl , 0.04 g.; glucose, 4.0 g.; Na acetate. $3\text{H}_2\text{O}$, 0.40 g.; dissolved in 400 ml. of tap water, and resuspended in 400 ml. of the same solution. The solution was adjusted to pH 5.5 with NaOH . This medium was modified from that used in earlier work as follows: (a) acetate was included as a carbon source; (b) $\text{NH}_4\text{H}_2\text{PO}_4$ replaced NH_4Cl as the nitrogen source in order to stabilize the pH further as the NH_3 was utilized during growth; (c) yeast and liver extracts were omitted in order to be able to define the carbon sources accurately.

The cell suspension was dispensed into the following flasks:

(1) 2 ml. were added to each of four Warburg manometer flasks. The O_2 taken up and the CO_2 evolved was measured by the direct method of Warburg (Umbreit, Burris & Stauffer, 1949).

(2) Three 1 l. Erlenmeyer flasks each received 100 ml. of cell suspension. In one flask (A) was suspended a small test-tube containing 200 $\mu\text{c.}$ of $\text{Ba}^{14}\text{CO}_3$ (23 mg.); this was so arranged that $^{14}\text{CO}_2$ could be released in the flask by the subsequent addition of acid. A second flask (B) received 100 $\mu\text{c.}$ of methyl-labelled Na acetate (1.6 mg.) and a third flask (C) 100 $\mu\text{c.}$ of carboxyl-labelled Na acetate (1.9 mg.). After mixing, small amounts of suspension were removed from the flasks containing labelled acetate, centrifuged, and the supernatants reserved for subsequent assay of the initial radioactivities in these media.

(3) Two 250 ml. Erlenmeyer flasks (D and E) each received 25 ml. of cell suspension.

All the Warburg and Erlenmeyer flasks were flushed with pure O_2 for 10 min. and then sealed. A preliminary experiment had shown that the O_2 in 900 ml. of air was insufficient to support the fully aerobic growth of 100 ml. of cell suspension for more than about 30 min. at the cell concentration used. Thereafter the R.Q. rose rapidly from about 1.1 to 3, or even higher. With an atmosphere of O_2 the R.Q. remained low and constant.

At the start of the experiment $^{14}\text{CO}_2$ was released in the flask containing $\text{Ba}^{14}\text{CO}_3$ by the addition of acid to the $\text{Ba}^{14}\text{CO}_3$. Warburg manometer readings were begun, and samples of the cell suspension were removed from one of the 250 ml. Erlenmeyer flasks (D) for dry-weight determinations. Samples were also taken from this flask for determinations, after hydrolysis, of the total amino acid content of the suspensions; these samples were immediately mixed with ethanol.

The various flasks were shaken for $4\frac{1}{2}$ hr. at 25° . At the end of this period, further samples of the suspension were removed from the two flasks containing labelled acetate, centrifuged, and the supernatants retained for assay of the residual radioactivities in the media. The bulk of the cell suspensions from the three 1 l. Erlenmeyer flasks was separately centrifuged, and each batch of cells was resuspended in 5% (w/v) trichloroacetic acid to kill the cells. Replicate samples of the cell suspension were removed from the other 250 ml. Erlenmeyer flask (E) for dry-weight measurements, and further samples were mixed with ethanol for the subsequent determination of the total amino acid content after hydrolysis of the proteins. A known weight of cells was also centrifuged and extracted with 70% (v/v) ethanol in water for determination of the free (non-protein) amino acids in the cells.

Dry-weight determinations. Samples of the cell suspension were filtered through weighed sintered glass crucibles and dried to constant weight at 105° .

Treatment of the radioactive cell material

In order to obtain a fraction containing all the insoluble cell nitrogen, each suspension of cells in trichloroacetic acid was treated as follows: after heating at 80° for 15 min., the suspension was centrifuged. The residue was washed with water, extracted with ethanol for 15 min. at 70° , washed with a mixture of equal parts of ethanol and ether, and finally washed with ether alone. The residue was dried to constant weight for some days in air at 37° ; the yield of extracted cells was about 300 mg. in each batch.

(a) *Isolation of glutamic and aspartic acids.* Each sample of extracted cells was hydrolysed in a sealed vessel at 105° for 18 hr. with a mixture of equal parts of 10N-HCl and glacial acetic acid. After evaporation to dryness, the residue was dissolved in water and a portion retained for chromatography and radioautography of the amino acids. The remainder was passed through a column of Deacidite E (prepared in the manner described by Partridge & Brimley (1949) for Deacidite B). Glutamic and aspartic acids were adsorbed on to the resin; they were eluted with N-HCl and separated from each other by paper chromatography using a solvent consisting of the organic layer of a mixture of *n*-butanol:acetic acid:water (4:1:5; Partridge, 1948). The amino acids were located by radioautography and eluted from the paper with water.

(b) *Isolation of lysine and arginine.* Part of the effluent from the Deacidite was chromatographed on paper for 7 days using *n*-butanol:acetic acid:water (4:1:5) as the solvent. Lysine and arginine were located by radioautography and eluted.

(c) *Isolation of isoleucine, proline and threonine.* Isoleucine, proline, threonine and a mixture of valine and methionine were isolated from another portion of the Deacidite effluent by paper chromatography with a solvent consisting of *n*-butanol:ethyl-methyl-ketone:8.5N-aqueous NH₃ (5:3:2; Wolfe, 1957). The amino acids were located as above and eluted.

(d) *Isolation of methionine.* The mixture of valine and methionine recovered from the previous chromatogram was oxidized with H₂O₂ and the oxidized methionine separated from valine by paper chromatography with *n*-butanol:acetic acid:water (4:1:5) as the solvent.

All samples of the amino acids isolated were subjected to paper chromatography in two solvents (the *n*-butanol:acetic acid:water, and *n*-butanol:ethyl-methyl-ketone:8.5N-aqueous NH₃ solvents used above) to check their purity and identity. To determine the specific activities of the various amino acids, measured samples were taken for radioactivity assays, and for amino acid determinations (Yemm & Cocking, 1955) after first drying *in vacuo* with 0.1N-NaOH (Fowden, 1951). Proline was estimated by the method of Chinard (1952).

(e) *Chromatography of the complete hydrolysate.* Samples of the hydrolysed protein were run on two-dimensional chromatograms with *n*-butanol:ethyl-methyl-ketone:8.5N-aqueous NH₃ as the first solvent, and *n*-butanol:acetic acid:water as the second. The chromatograms were exposed to X-ray film and subsequently sprayed with an 0.05% (w/v) solution of ninhydrin in ethanol.

All these procedures were carried out for the contents of each of the flasks A, B and C.

Determination of the total (free and combined) amino acids. The samples of the whole cell suspensions mixed with ethanol taken at the beginning and end of the experiment (from flasks D and E respectively) were evaporated to dryness. The proteins were hydrolysed as described above and the amino acids were adsorbed on to a column of Zeocarb 215 (Partridge & Brimley, 1952). After elution with 2N-NH₃ the amino acids were chromatographed and determined as described by Wolfe (1957).

Determination of the free intracellular amino acids. The sample taken for this purpose from flask E at the end of the experiment was extracted three times with 70% (v/v) ethanol in water for 15 min. at 70°. After centrifugation the pooled extracts were evaporated to dryness and the amino acids redissolved in water, adsorbed on to Zeocarb 215, eluted with NH_3 , and chromatographed as described above. The dried chromatograms were sprayed with ninhydrin and the amounts of the various amino acids present were roughly determined by visual comparison with standard quantities.

Degradation of glutamic and aspartic acids. Samples of each acid were decarboxylated with lyophilized *Clostridium welchii* (Meister, Sober & Tice, 1951) to obtain the α -carboxyl carbons as CO_2 ; in addition the aspartic acid samples were decarboxylated with ninhydrin (van Slyke, MacFadyen & Hamilton, 1941) to obtain both carboxyl carbons as CO_2 . The various products obtained were assayed for radioactivity. The activities of the β -carboxyl carbons of the aspartic acids were obtained by difference.

Radioactivity determinations. Radioactivity was measured by the 'infinitely thin' sample method of Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949, p. 107). The solution to be counted (0.05 ml.) was pipetted on to a recessed aluminium planchette, 2.1 cm. in diameter. The drop was covered with a circle of lens tissue paper, the sample dried under an infrared lamp at about 70° for 4 min., and counted in a windowless gas flow (methane) proportional counter connected to a scaler.

Samples containing $^{14}\text{CO}_2$ absorbed in N-NaOH were not converted to the Ba salt but were applied directly to the planchettes. There was no detectable loss of $^{14}\text{CO}_2$ by this method (see also Calvin *et al.* 1949, p. 122).

Nelson & Krotkov (1955) determined the activities of samples of labelled glucose, asparagine and glutamine before and after conversion to BaCO_3 and found no great difference in specific activities by the two methods.

In order to determine specifically the activity due to acetic acid, acetate samples were counted after drying in the presence of a drop of 2N-HCl (which released the acetic acid) or 2N-NaOH (which retained it).

As the samples possessed appreciable mass, correction was made for the self-absorption of the radiation by the NaOH, lens tissue and sample itself.

Chemicals. All isotopic compounds were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Growth and respiration

The details of the increases in gas exchange rates and dry weights, and the utilization of radioactive substrates in the medium during the experimental growth period are given in Table 1. These values refer to the total contents of each of the 1 l. flasks A, B and C used for the growth of the cells on radioactive substrates. There was good agreement between the percentage increases of the rates of gas exchange and of the dry weights.

The rates of oxygen absorption and carbon dioxide evolution at intervals during the course of the experiment, together with the R.Q., are shown in

Fig. 1. The increases of the rates of gas exchange were linear for both gases, resulting in very little change of R.Q. over the whole experiment.

During the experimental period methyl- and carboxyl-labelled acetate was almost completely metabolized; there was some small excretion of non-volatile labelled substances into the medium (Table 1). Of the total radioactivities in flasks B and C at the start of the experiment, only 1.8 and 2.6 %, respectively, were not volatile when dried in the presence of acid, i.e. were not acetate. At the end of the experiment these values had risen to 96.1 and 95.7 % of the activities remaining at this time, though the total activities in the media at the end of the experiment were very much reduced. The activity of the labelled carbon dioxide in flask A was not measured.

Table 1. *Rates of gas exchange, dry weights, and radioactivity in the medium of cell suspensions growing in the presence of labelled carbon dioxide and acetate*

Three 1 l. Erlenmeyer flasks each contained 100 ml. of a suspension of growing cells, the gas phase being pure O_2 . One flask was supplied with $^{14}CO_2$, a second with $^{14}CH_3.COOH$, and a third with $CH_3.^{14}COOH$, in addition to unlabelled substrates which were identical in each case. The values refer to the totals in each of the three flasks. The cells were grown for $4\frac{1}{2}$ hr. at 25° .

	Start of growth period	End of growth period	Final value as % of initial value
Rate of O_2 uptake (ml./hr.)	16.60	40.40	243.4
Rate of CO_2 evolution (ml./hr.)	17.36	45.98	264.9
Q_{O_2} (μ l./hr./mg. dry wt.)	93.3	91.6	—
Q_{CO_2} (μ l./hr./mg. dry wt.)	97.5	104.3	—
R.Q.	1.046	1.138	—
Dry weight (mg./flask)	178.0	441.0	247.8

Radioactivity in the medium (counts/min.)

(a) Suspension supplied with $^{14}CH_3.COOH$:

Medium + NaOH	60,357,000	3,787,000	—
Medium + HCl	1,089,000	3,640,000	—
Thus activity due to acetate	59,268,000	147,000	0.2

(b) Suspension supplied with $CH_3.^{14}COOH$:

Medium + NaOH	80,671,000	3,864,000	—
Medium + HCl	2,111,000	3,699,000	—
Thus activity due to acetate	78,560,000	165,000	0.2

Total synthesis of amino acids

Table 2 shows the total quantities of amino acids in the cell suspensions after hydrolysis. The differences between the values for the beginning and end of the experiment show the net quantities of amino acids synthesized in each of the three growth flasks containing labelled substrates. It will be noted that there was not the same percentage increase in each of the amino acids during the period of the experiment. This applied particularly to lysine, i.e. the cells contained relatively more lysine at the beginning of the experiment than at the end. This may be a reflexion of the fact that a fungal hypha grows only at its tips, so that a difference in amino acid composition is understandable between old growth and new growth when cells are grown in different conditions. In

the case in question, the germination of the spores and the initial growth of the mycelium took place in the medium used in earlier work (Moses, 1954), and in an atmosphere of air; subsequent growth was in a modified medium in an atmosphere of oxygen. On the whole, however, there was good agreement

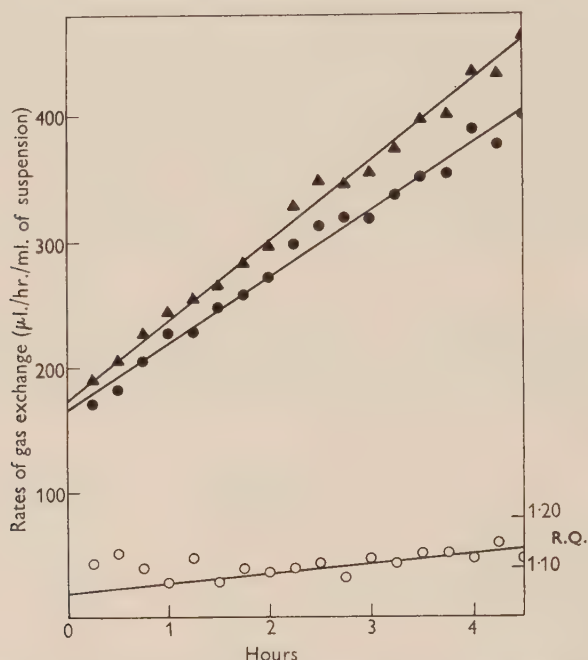


Fig. 1. R.Q. values and rates of gas exchange of growing cells. Each flask contained 100 ml. of cell suspension containing initially 178 mg. dry wt. of cells. The cells were allowed to grow for 4.5 hr. at 25° in an atmosphere of pure O₂. The gas exchange is expressed as μl./hr./ml. of suspension. ▲—▲, carbon dioxide evolution; ●—●, oxygen uptake; ○—○, R.Q.

Table 2. *Synthesis of amino acids during growth of the cell suspensions*

Three flasks each contained 100 ml. of a suspension of growing cells, the three suspensions differing only in the labelling with ¹⁴C of some of the substrates. Samples of the suspensions were taken before and after a 4½ hr. growth period for determination, after hydrolysis of the cells, of the content of several amino acids in the whole suspensions. The amounts of these amino acids synthesized during the growth period were determined by difference. The values given refer to the total amino acid content of the cell suspensions in each flask.

Amino acid	Initial quantity before growth (μmole)	Final quantity after growth (μmole)	Quantity synthesized (μmole)	Percent increase during growth
Arginine	13	37	24	291
Aspartic acid	67	169	102	252
Glutamic acid	77	216	139	279
Isoleucine	34	89	55	265
Lysine	135	153	18	113
Methionine	8	23	15	276
Proline	42	103	61	248
Threonine	60	151	91	252

between the percentage increases of most of the amino acids measured, both among themselves, and when compared with the percentage increase in dry weight of the cells.

Free amino acids present in the cells

Twenty ninhydrin-reacting substances were present in hot 70% ethanol-extracts of the cells. Seventeen were identified as amino acids or amides; the other three were present in trace amounts only. Asparagine was absent. Many of the amino acids, and particularly alanine and arginine, were present in fairly large quantities; others were found in lower concentrations (Table 3). Since the nitrogen content of the cells was about 8% of the dry weight (Moses, 1954), the free amino acids constituted about 3% of the total cell nitrogen.

Table 3. *Free amino acids present in a hot 70% (v/v) ethanol extract of growing cells*

Samples of a growing cell suspension were centrifuged and the cells (7.5 mg. dry wt.) extracted with hot 70% (v/v) ethanol. The free amino acids present were chromatographed and estimated visually by comparison with standard spots after spraying the chromatograms with ninhydrin: there may be an error of $\pm 100\%$. The values given are in $\mu\text{g.}$ of α -amino nitrogen.

Amino acid	Quantity present ($\mu\text{g.}$ of α -amino N)	Amino acid	Quantity present ($\mu\text{g.}$ of α -amino N)
Alanine	5	Lysine	0.5
γ -Aminobutyric acid	1	Methionine + valine	1
Arginine	1.5	Phenylalanine	0.5
Aspartic acid	0.5	Proline	0.5
Cystine or cysteine	trace	Serine	1
Glutamic acid	0.5	Threonine	trace
Glutamine	0.5	Tyrosine	1
Glycine	1	Unknown 1	trace
Isoleucine	trace	Unknown 2	trace
Leucine	1	Unknown 3	trace

Incorporation of labelled carbon into the amino acids of protein

Isotopic carbon was incorporated into some, though not all, of the amino acids of the cellular protein from cells grown on the three tracer substances supplied. Cells supplied with labelled carbon dioxide showed strong radioactivity in aspartic and glutamic acids, arginine, proline, methionine, isoleucine, and threonine, with traces in alanine, valine and glycine. When the cells were grown on either methyl- or carboxyl-labelled acetate the distribution of activity among the various amino acids was nearly identical, suggesting that acetate was incorporated largely as an intact molecule. Activity was found in considerable quantities in both cases in aspartic and glutamic acids, arginine, proline, methionine, isoleucine, threonine, leucine, and lysine, and in an unidentified substance which did not react with ninhydrin. Traces were seen in alanine. Cells supplied with methyl-labelled acetate showed some activity in valine; those grown on carboxyl-labelled acetate appeared not to possess activity in valine, though in this case the chromatographic separation between valine and methionine was poorer than in the other instances. Photographs of the amino acid chromatograms after

being sprayed with ninhydrin, together with the corresponding radioautograms, are shown in Pl. 1.

The specific activities of a number of amino acids from each sample of hydrolysed protein were determined and are shown in Table 4.

Table 4. *Specific activities of various amino acids isolated from cells growing on labelled substrates*

Eight amino acids were isolated from cells growing in a salts + NH_4^+ + glucose + acetate medium in the presence of one of three labelled substrates: $^{14}\text{CO}_2$, $^{14}\text{CH}_3\cdot\text{COOH}$, $\text{CH}_3\cdot^{14}\text{COOH}$. The specific activities of the amino acids were determined and are expressed as counts/min./ μmole .

Amino acid	Tracer supplied		
	$^{14}\text{CO}_2$	$^{14}\text{CH}_3\cdot\text{COOH}$	$\text{CH}_3\cdot^{14}\text{COOH}$
Aspartic acid	24,100	12,100	9,500
Methionine	29,300	10,800	9,100
Threonine	28,400	21,100	19,600
Isoleucine	28,000	16,800	16,500
Glutamic acid	9,300	30,100	25,800
Proline	14,800	36,100	31,500
Arginine	33,000	24,800	22,700
Lysine	3,700	45,600	38,300

Degradation of aspartic and glutamic acids

Table 5 shows the intramolecular distribution of activity in the three aspartate and glutamate samples isolated.

Table 5. *Distribution of radiocarbon in the aspartic and glutamic samples isolated from cells grown in the presence of labelled substrates*

Samples of glutamic and aspartic acid were isolated from cells growing in the presence of $^{14}\text{CO}_2$, $^{14}\text{CH}_3\cdot\text{COOH}$, or $\text{CH}_3\cdot^{14}\text{COOH}$. The amino acids were partially degraded and the specific activities of certain carbon atoms determined. The activities are expressed both as counts/min./ μmole , and as a percentage of the activity in the complete molecule.

	Labelled substrate					
	$^{14}\text{CO}_2$		$^{14}\text{CH}_3\cdot\text{COOH}$		$\text{CH}_3\cdot^{14}\text{COOH}$	
	Counts/min.	%	Counts/min.	%	Counts/min.	%
Aspartic acid						
COOH	18,297	76.0	1,761	14.6	4,696	49.7
$\left. \begin{array}{l} \text{CH}_2 \\ \text{CHNH}_2 \end{array} \right\}$	1,099	4.5	8,855	73.4	798	8.4
COOH	4,680	19.5	1,440	11.9	3,963	41.9
Total	24,076	100.0	12,056	100.0	9,457	100.0
Glutamic acid						
COOH	8,451	91.3	2,020	6.7	5,964	23.1
$\left. \begin{array}{l} \text{CHNH}_2 \\ \text{CH}_2 \\ \text{COOH} \end{array} \right\}$	804	8.7	28,031	93.3	19,824	76.9
Total	9,255	100.0	30,051	100.0	25,788	100.0

DISCUSSION

The operation of the citric acid cycle

From a theoretical consideration of the citric acid cycle, certain predictions can be made regarding the intramolecular distribution of radioactivity in some of the cycle products when the labelled substrates provided are marked in certain specified atoms only. It is possible to predict, in particular, the labelling of α -ketoglutaric and oxalacetic acids, and assuming that these keto acids are in equilibrium with their corresponding amino acids, the same labelling pattern should hold also for the latter. Close agreement between theoretical prediction and observed fact is good support for the real existence and operation of a proposed system.

When cells are supplied with ^{14}C -carbon dioxide, the incorporation of radioactivity should be relatively greater in aspartic acid and its family of amino acids than in the glutamic acid family. All the radiocarbon should be confined to the carboxyl carbons of aspartic acid, and to the α -carboxyl carbon of glutamic acid. The activities of the α -carboxyl carbon of glutamic acid and the β -carboxyl carbon of aspartic acid should be similar. Tables 4 and 5 show that with the exception of the last criterion these conditions were broadly fulfilled by cells of *Zygorrhynchus moelleri* growing in the presence of labelled carbon dioxide. There was evidence of a lack of complete equilibration of radio-carbon, due probably to the presence of the free amino acid pool (see below). This has resulted in a closer agreement between prediction and observation in the distribution of activity within a particular molecular species, than to the relative activities of two different chemical substances. Thus the label was virtually confined to the relevant carboxyl carbons of aspartic and glutamic acids, although the activity of the aspartic β -carboxyl carbon was greater than that of the glutamic α -carboxyl carbon (Tables 4 and 5).

With methyl-labelled acetate, the two carboxyl carbons of aspartic acid should be equally active, and should each have the same specific activity as the α -carboxyl carbon of glutamic acid. The specific activities of the glutamic family of amino acids should be greater than those of the aspartic family. These considerations also hold for carboxyl-labelled acetate and, in addition, with this tracer substance all the activity in both aspartic and glutamic acids should be confined to the carboxyl carbons. The predictions were broadly confirmed in cells grown on these two species of labelled acetate (Tables 4 and 5).

There can therefore be little doubt that not only can the individual reactions of the tricarboxylic acid cycle be carried out by *Zygorrhynchus moelleri* (Moses, 1955), but the cycle does in fact operate as an organized unit during the growth of the organism. Roberts *et al.* (1955, chapter 14) have described methods by which the amount of recycling taking place in the cycle can be calculated from the distributions of radioactivity in aspartic and glutamic acids when the cells are grown on various substrates. In a steady-state system in growing cells the Krebs cycle is assumed to have a twofold function: oxidation of acetate and synthesis of amino acids. In its oxidative

capacity the cycle is purely catalytic, and each molecule of oxalacetic acid entering the cycle by condensation with acetate to form citrate is replaced after one turn of the cycle. For the synthesis of amino acids, however, one molecule of oxalacetate will be lost from the cycle for each molecule of amino acid synthesized, and a fresh molecule must be synthesized from pyruvate and carbon dioxide. If the organisms are supplied with labelled acetate, it can be shown that whereas oxalacetate which has travelled round the cycle is labelled in certain carbon atoms, newly formed oxalacetate will be unlabelled. Thus the extent of labelling within the molecule will be dependent on the percentage of oxalacetate utilized in citrate synthesis which is formed from recycled C_4 -dicarboxylic acid (K) and on the percentage synthesized *de novo* from pyruvate and carbon dioxide (P). By measuring the ratios of labelling in the various carbon atoms of glutamic and aspartic acids, and assuming that these reflect the labelling pattern in the corresponding keto acids, it is possible to calculate the ratio P/K , either by comparing the specific activities of carbon atoms within each glutamic or aspartic acid molecule, or by comparing the specific activities of the total glutamic and aspartic acid molecules. Table 6 shows some of the values calculated for the P/K ratio in *Z. moelleri*; for the reasons advanced above, the most reliable estimates are those derived from intra- rather than from inter-molecular activities. It is probable that the value for P/K was about 40/60.

Table 6. *Degree of recycling in the tricarboxylic acid cycle in growing cells*

Cells were grown in the presence of $^{14}CO_2$, $^{14}CH_3.COOH$, or $CH_3.^{14}COOH$. Samples of glutamic and aspartic acid were isolated from each batch of cells, the amino acids were partially degraded, and the specific activities of certain of their carbon atoms were determined. From the ratios of the specific activities of particular carbon atoms the value of P/K was estimated, where P is the % of oxalacetate synthesized from pyruvate and carbon dioxide at the start of each turn of the citric acid cycle, and K is the % of recycled C_4 acid (for explanation see text).

Method of calculation	P/K
Cells grown in the presence of $^{14}CH_3.COOH$:	
1. Ratio of specific activities of carbons (1+4)/(2+3) of aspartic acid	27.7/72.3
2. Ratio of specific activities of carbons 1/(2+3+4+5) of glutamic acid	38.6/61.4
3. Ratio of specific activities of total glutamic acid/total aspartic acid	58.5/41.5*
Cells grown in the presence of $CH_3.^{14}COOH$:	
4. Ratio of specific activities of carbons 1/(2+3+4+5) of glutamic acid	39.8/60.2
5. Ratio of specific activities of total glutamic acid/total aspartic acid	55.1/44.9*

* These values are probably less accurate due to incomplete equilibration of the radio-carbon between glutamic and aspartic acids (see text).

Pathways of amino acid biosynthesis

On the basis of their studies with *Escherichia coli*, Abelson, Bolton, Britten, Cowie & Roberts (1953), and McQuillen & Roberts (1954) found that certain amino acids had very similar specific activities suggesting an intimate metabolic relation between them. A number of amino acid families were defined and it was supposed that the carbon skeletons of the amino acids in a particular

family were derived undiluted from the same precursor. Thus, in *E. coli* grown on labelled acetate, the amino acids aspartic acid, lysine, methionine, threonine and isoleucine all had similar specific activities and were described as the aspartic family; similarly, the glutamic family included glutamic acid, proline and arginine. The same considerations held for cells grown in the presence of labelled carbon dioxide, except that the specific activity of arginine was much greater than that of proline and of glutamic acid. It was found that the carbon skeleton of the ornithine moiety of arginine originated from glutamate, and the guanidine carbon from carbon dioxide; in this case, therefore, the carbon dioxide contributed additional activity to the arginine molecule.

Although the pattern of specific activities was broadly similar, the results with *Zygorrhynchus moelleri* were not quite so conclusive. When the specific activity of aspartic acid was high (e.g. when the cells were given labelled carbon dioxide) the activities of threonine, methionine and isoleucine were also high (Table 4), and similarly the values were lower when the aspartate values were lower (cells grown on labelled acetate). Similar relationships existed between glutamic acid, proline and arginine, though here the activities were highest in labelled acetate-grown cells and lowest in those grown with labelled carbon dioxide. The results thus confirm the equivalent findings in *Escherichia coli*.

Lysine, a member of the aspartic family in *Escherichia coli*, was very weakly labelled in *Zygorrhynchus moelleri* grown on ^{14}C -carbon dioxide, although aspartic acid was highly active. When methyl- or carboxyl-labelled acetate was used, lysine was far more active than any other amino acid (Table 4). Lysine was evidently not a member of the aspartic family, and from its high specific activity in cells grown on acetate tracer it appeared to arise from the succinate moiety of α -ketoglutarate together with acetate (or a related compound) which was in equilibrium with exogenous or cycle acetate (Davis, 1955). The pathway appeared to be similar to that operating in *Torulopsis utilis*, but different from those of *Neurospora crassa*, where the acetate portion is not in equilibrium with exogenous acetate (Abelson & Vogel, 1955), and of *Escherichia coli*, where lysine is derived from aspartate (Roberts *et al.* 1955).

In *Escherichia coli* the agreement between the specific activities of the members of each amino acid family was very good, and in this organism no free amino acid pool was found (Roberts *et al.* 1955, p. 16). In *Zygorrhynchus moelleri*, however, the correlation was less precise and a considerable amino acid pool was found to be present (Table 3): it seems probable that the presence of this pool prevented complete equilibration of radioactivity among the various amino acids of each family.

Quantitative significance of the tricarboxylic acid cycles

From a knowledge of the various amino acids synthesized in the course of the experiment (Table 2), it can be calculated that the products of the citric acid cycle consisted of 263 μmole of aspartic acid (for the production of aspartic acid, methionine, threonine and isoleucine), 224 μmole of glutamic acid (for glutamic acid, proline and arginine synthesis), and 18 μmole of

lysine. There may also have been other unknown products. These values, together with a P/K ratio of 40/60, enable a flow diagram to be constructed for the various branches of the tricarboxylic acid cycle (Fig. 2). From these figures, 505 μ mole of oxalacetate must enter the cycle to maintain the steady state conditions and replace the 505 μ mole of amino acids removed from the cycle. As the P/K ratio is 40/60 (Table 6), the amount of recycled oxalacetate is 783 μ mole. The proportions of aspartic acid arising from oxalacetate by an

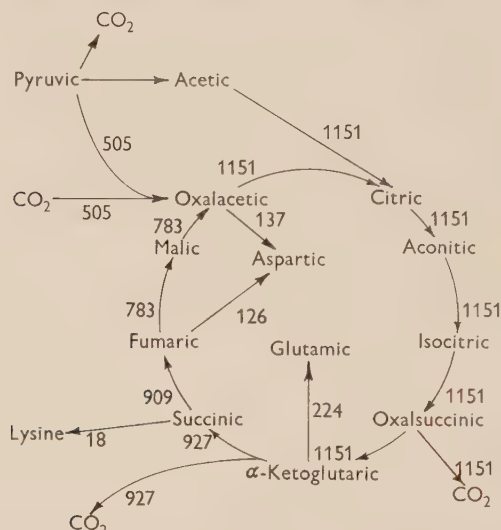


Fig. 2. Flow rates in the tricarboxylic acid cycle of growing cells. The values given are the total flow rates (as μ mole/4.5 hr.) over a 4.5 hr. period during which the dry wt. of the cells increased from 178 to 441 mg.

asymmetrical (direct) pathway and by a symmetrical pathway (probably via malate and fumarate) can be calculated from the distribution of radiocarbon between the two carboxyl carbons of aspartic acid from cells grown on labelled carbon dioxide (Roberts, Cowie, Britten, Bolton & Abelson, 1953). Thus 126 μ mole of aspartate arise from fumarate and 137 μ mole from oxalacetate, so that $505 + 783 - 137 = 1151$ μ mole of oxalacetate are available for citrate formation. For synthetic purposes 224 μ mole of glutamic acid are removed, followed by 18 μ mole of lysine and 126 μ mole of aspartic acid, leaving 783 μ mole of recycled oxalacetic acid; the quantity of carbon dioxide produced by cycle decarboxylations can also be deduced, as well as the amount required for the synthesis of fresh oxalacetate from pyruvate. These represent minimum flow rates, as other compounds not investigated in the present work may also arise from the cycle. The flow rates in the different cycle reactions in growing *Zygorrhynchus moelleri* were about 16% of those in growing *Escherichia coli*.

During the course of the experiment 6362 μ mole of carbon dioxide were evolved in each of the three growth flasks (calculated from the data of Table 1 and Fig. 1). The citric acid cycle produced 1151 μ mole by the decarboxylation

of oxalsuccinate, 927 μ mole by α -ketoglutarate decarboxylation, and used 505 μ mole for the synthesis of new oxalacetate to replace that diverted into amino acid manufacture; the net production of carbon dioxide from the cycle was thus 1573 μ mole, or 24.7% of the total.

Comparison with other organisms

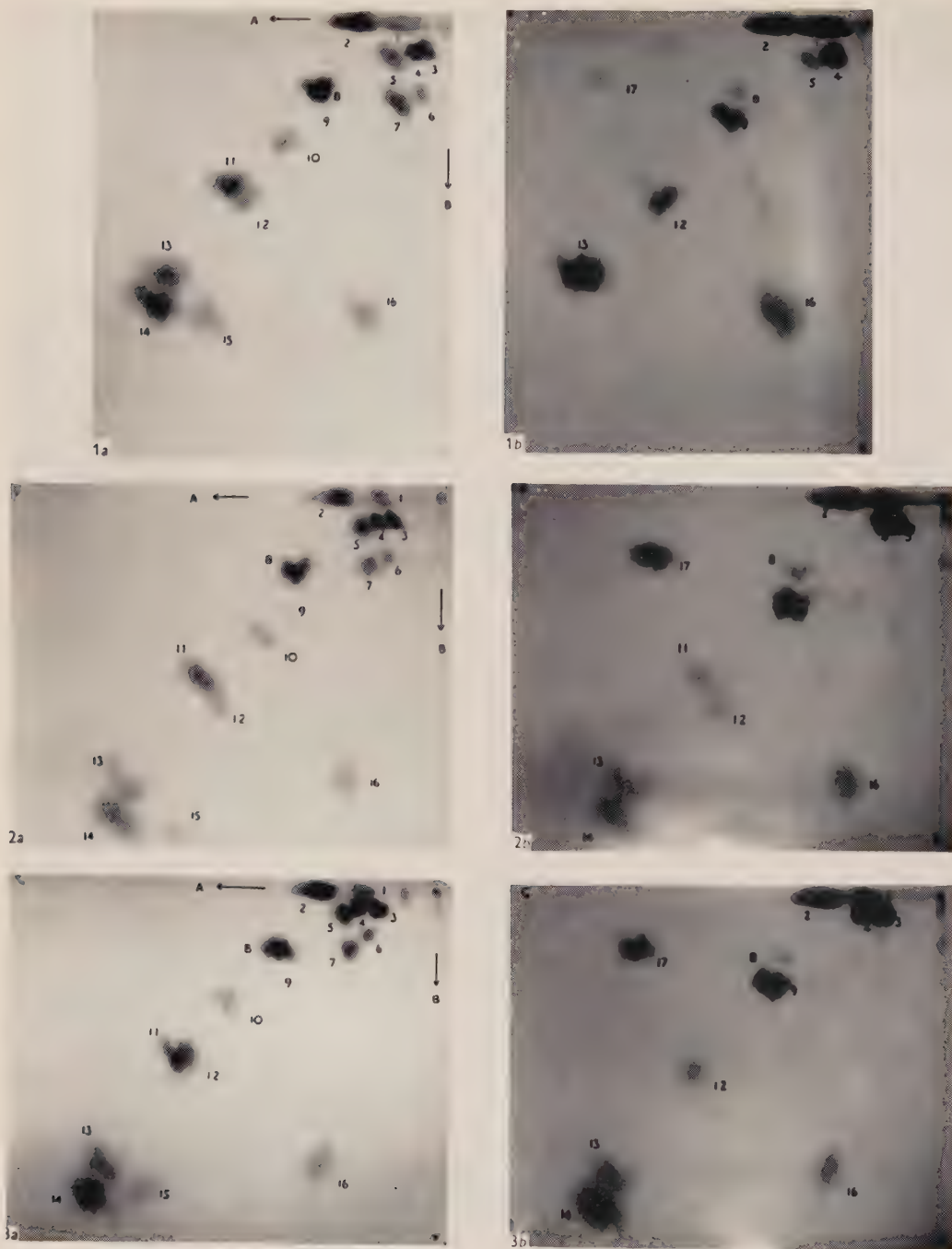
These results confirm that whilst in this organism the cycle largely serves to supply intermediates for organic syntheses (Krebs, Gurin & Eggleston, 1952), it nevertheless also plays an important part in the terminal oxidation mechanisms and the production of respiratory carbon dioxide. Lewis & Weinhouse (1951) found with *Aspergillus niger* that the distribution of isotopic activity in citric acid from cells metabolizing carboxyl-labelled acetate was consistent with the cycle being a major pathway for acetate utilization and citrate formation. Similar conclusions were reached by Yall (1955) from the results of supplying labelled acetate to resting cells of *Penicillium chrysogenum*. Butterworth, Gilmour & Wang (1955) found a C_3 - C_1 condensation together with Krebs cycle activity to be a major pathway of carbon dioxide fixation in *Streptomyces griseus*, and from the distribution of activity in glutamic acid synthesized by cells of the same organism supplied with carboxyl-labelled acetate (Gilmour, Butterworth, Noble & Wang, 1955), the P/K ratio can be calculated as 10/90. In *Escherichia coli* the ratio was about 70/30, and only 2.4% of the respiratory carbon dioxide released originated in the Krebs cycle.

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V. MOSES—KREBS CYCLE FLOW RATES IN A GROWING FUNGUS. PLATE 1

(Facing p. 549)

EXPLANATION OF PLATE

Photographs of the chromatograms (after being sprayed with ninhydrin) and the corresponding radioautograms of the protein hydrolysates from cells growing on one of three labelled substrates.

1. Cells grown on labelled carbon dioxide. (a) chromatogram; (b) radioautogram.
2. Cells grown on methyl-labelled acetate. (a) chromatogram; (b) radioautogram.
3. Cells grown on carboxyl-labelled acetate. (a) chromatogram; (b) radioautogram.

Key: 1, aspartic acid; 2, glutamic acid; 3, lysine; 4, arginine; 5, glycine; 6, histidine; 7, serine; 8, alanine; 9, proline; 10, tyrosine; 11, valine; 12, methionine; 13, isoleucine; 14, leucine; 15, phenylalanine; 16, threonine; 17, unknown (not ninhydrin-reactive). Solvent directions: *A*, *n*-butanol:acetic acid:water; *B*, *n*-butanol:ethyl-methyl-ketone:8.5*N*-aqueous NH_3 .

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The Pathway of Glucose Metabolism in *Zygorrhynchus moelleri*

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SUMMARY: Radioactive glucose has been supplied to starved and unstarved cells of the fungus *Zygorrhynchus moelleri* for short periods of time, and a partial analysis was made of the nature of those substances incorporating radiocarbon from labelled glucose. It was found that although glucose was metabolized more rapidly by unstarved cells it rapidly entered the cells in both nutritional states and was converted to a number of other substances within the first 15 sec. Differences were found in the pattern of incorporation of labelled carbon between starved and unstarved cells, suggesting that starvation resulted in a disturbance of the balance of the relative metabolic pathways utilized for glucose metabolism in unstarved cells.

In further experiments cells were prepared in four physiological states: growing, resting after growth, starved in buffer, and after metabolizing glucose for several hours subsequent to starvation. By use of substrates variously labelled with radiocarbon it was found that the extent of recycling in the tricarboxylic acid cycle, which is similar in growing and resting cells, rose considerably when the cells were starved, and remained high for several hours during the incubation of starved cells with glucose. The minimum percentage of glucose which was metabolized primarily at the C₁ position rose from 11 % in growing cells to 19 % in resting cells, fell nearly to zero immediately following starvation, and increased to 5 % after some hours in the presence of glucose.

Cells of the fungus *Zygorrhynchus moelleri*, when starved for 24 hr. in phosphate buffer and then supplied with glucose, show a lag period of 2-3 hr. before the rates of respiration and glucose utilization become constant at their maximum values (Moses, 1954, 1955*a*). There is no appreciable lag with unstarved cells. Some evidence has been adduced (Moses, 1955*b*) suggesting that this lag may be due to some extent to a partial metabolic blockage on the pathway of glucose metabolism. A further investigation of the mechanism of glucose metabolism in this fungus is made in the present work by the use of two different types of technique.

In the first case starved and unstarved cells were allowed to metabolize uniformly labelled glucose for short periods of time. Attempts were then made to determine the nature of those compounds incorporating ¹⁴C from labelled glucose and to investigate differences in glucose metabolism exhibited by cells in the two different physiological conditions.

In the second series of experiments cells from four physiological states were used: growing cells, resting unstarved cells, starved cells, and starved cells which had been allowed to metabolize glucose for 3 hr. Experiments were carried out to assess the relative extents in the cells of recycling within the

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tricarboxylic acid cycle and of the percentage of glucose metabolized via an asymmetrical oxidative route rather than by a symmetrical pathway such as the Embden–Meyerhof glycolysis scheme.

METHODS

Incorporation of radioactivity from (^{14}C)glucose. Fungal hyphae were grown and, when required, starved for 24 hr. in 0.067 M-phosphate buffer, pH 6.8, as described by Moses (1954). The cells, starved or unstarved, were suspended in distilled water at a concentration of 12 mg. dry wt./ml., dispensed into a number of flasks in 5 ml. quantities and shaken at 25°. To each flask was added 1 ml. of a solution of uniformly labelled glucose (12.5 μC .; 160 μg .). After incubation in the presence of (^{14}C)glucose for 15 sec., 45 sec., 2 min., 6 min., and, in the case of starved cells, 20 min., the cells were killed by the addition of 20 ml. of boiling ethanol and were subsequently extracted at 80° for a further 30 min. The cell debris was removed by centrifugation and the ethanol extract evaporated to dryness *in vacuo* at room temperature.

The residue was dissolved in water and separated into three fractions by ion-exchange chromatography using the resins Amberlite IR-120 (in the H form) and Amberlite IR-4B (in the OH form) (Claridge & Werkman, 1954). The 'cationic' fraction, retained by and subsequently eluted from IR-120, contained inorganic cations and amino acids; the 'anionic' fraction contained inorganic anions and organic acids retained by and subsequently eluted from IR-4B; malate and citrate were not absorbed by this resin, and appeared with uncharged substances in the 'non-ionic' fraction. Each fraction was assayed for radio-activity by methods previously described (Moses, 1957) and was also subjected to two-dimensional paper chromatography using 80 % (v/v) phenol in water in the presence of NH_3 as the solvent for the first dimension, and butyric acid:butanol:water (2:1:1) for the second dimension (Aronoff & Vernon, 1950). Radioactive substances were located on the chromatograms by radioautography.

The identity of some of the radioactive substances present in the cell extracts was determined, whilst the nature of others remains unknown. Glutamic, succinic, malic, and citric acids, glucose, glucose-1-phosphate, and fructose-1:6-diphosphate were first tentatively identified from their positions on the chromatograms. The spots of these substances were cut out, eluted with water, and co-chromatographed with authentic samples of the suspected compounds. All substances were chromatographed in the two-dimensional solvent system used above, and use was also made of the solvent systems mentioned below. Identity of the radioactive area with the exact shape and position of the known marker after spraying, in several solvent systems, was taken as the criterion of identification of a substance.

The solvent systems used for the investigation of the active substances were as follows: organic acids were run in *tert*-amyl alcohol:chloroform:water:98 % (v/v) formic acid (8:8:8:3; L. E. Bentley, personal communication), and located by spraying the dried chromatograms with mixed methyl-yellow-bromophenol

blue indicator (Claridge & Werkman, 1954). Amino acids were chromatographed in 80% (v/v) aqueous phenol in an atmosphere of NH_3 and located with ninhydrin. Sugars and other reducing substances were run in a mixture of methanol:ethanol:water (2:2:1; Norris & Campbell, 1949), and were detected by an ammoniacal AgNO_3 spray (Trevelyan, Procter & Harrison, 1950). Phosphate esters were chromatographed on washed filter-paper in a solvent containing 90% (v/v) formic acid:isopropyl ether (2:3; Hanes & Isherwood, 1949). The esters were detected by the technique of Bandurski & Axelrod (1951), modified from that of Hanes & Isherwood (1949).

Incorporation of radioactivity from carboxyl-labelled acetate. Hyphae were grown for 18 hr. in 150 ml. of medium. The cells were harvested, washed and resuspended in 150 ml. of 0.067 M-phosphate buffer, pH 6.8. The cell suspension was divided into three equal parts.

Sample I was supplied with 0.02 M-glucose and shaken for 3 hr. at 25°. The cells were then centrifuged and resuspended in 50 ml. of 0.067 M-phosphate buffer (pH 6.8) containing 0.02 M-glucose and 0.002 M-Na acetate in a 500 ml. Erlenmeyer flask. Carboxyl-labelled acetate (0.44 mg.; 28 $\mu\text{c.}$) was added and a small sample of the suspension taken for assay of the initial radioactivity in the medium; the cells were shaken for 1 hr. at 25°, then centrifuged and resuspended in ethanol. Simultaneously with the above experimental arrangement monitor Warburg flasks were set up containing 2 ml. of cell suspension and corresponding quantities of labelled and unlabelled substrates, together with 0.2 ml. of N-KOH in order to trap respiratory CO_2 . Samples of the medium and KOH were removed at the end of the experiment for radioactivity determinations. The gas phase was air.

Cells of sample II were starved by being shaken in buffer for 24 hr. They were then centrifuged and resuspended in buffer; substrates were added and Warburg flask monitors set up exactly as in the case of sample I. The cells were shaken for 1 hr. after the addition of substrates; they were then treated with ethanol and samples taken as before.

Cells of sample III were starved in buffer for 24 hr., and then supplied with 0.02 M-glucose for 3 hr. The cells were finally centrifuged, resuspended in buffer and substrates were added as described for the other two samples. These cells were also shaken for 1 hr. before samples of the medium and KOH were removed and the cells suspended in ethanol.

The ethanolic suspension of each batch of cells was evaporated to dryness. The residue was hydrolysed and glutamic acid isolated from each batch of hydrolysate. The specific radioactivity of each glutamic acid sample was determined; each sample of glutamic acid was also decarboxylated with *Clostridium welchii* S.R. 12, and the specific activity of the α -carboxyl carbons measured. The techniques employed have been described elsewhere (Moses, 1957).

Respiration of labelled glucose. The production of respiratory $^{14}\text{CO}_2$ from (^{14}C)glucose and from ($^{14}\text{C}_1$)glucose was investigated in a further series of experiments. Three samples of cells in different physiological states were prepared, corresponding to samples I, II and III in the previous section.

A further sample (IV) was prepared corresponding to the growing cells used in an earlier investigation (Moses, 1957). The latter were grown for 13 hr. only instead of the customary 18 hr., and were subsequently resuspended in a modified growth medium containing glucose and acetate as carbon source, and shaken in an atmosphere of pure O_2 (Moses, 1957).

Cells of sample I were allowed to metabolize 0.02M-glucose for 3 hr. after growth as in the first experiment. After resuspension in 50 ml. of 0.067M-phosphate buffer, pH 6.8, the cells were dispensed into Warburg flasks in 2 ml. quantities. Glucose labelled with ^{14}C either uniformly or in the C_1 position (2 $\mu c.$) was added to each flask to give a final concentration of 0.002M. KOH was added to the centre wells to trap the $^{14}CO_2$ evolved, and the cells were shaken for 1 hr. at 25°. A similar experiment was conducted in which the cells, having metabolized glucose for 3 hr. after growth, were placed in an atmosphere of pure N_2 and allowed to ferment the two species of labelled glucose supplied. The $^{14}CO_2$ evolved was again trapped in KOH.

The starved cells of sample II were supplied with labelled glucose immediately after being shaken in buffer for 24 hr. The respiratory $^{14}CO_2$ was collected for 1 hr. at 25°.

With sample III, labelled glucose was supplied to the starved cells for 1 hr. following a preliminary 3 hr. period for the metabolism of unlabelled glucose (0.02M). In all these instances the gas phase was air.

The cells from the modified growth medium (sample IV) were incubated for 1 hr. at 25° in an atmosphere of O_2 . The labelled glucoses were then added, and the cells shaken for 1 hr. longer in O_2 .

In each case samples of the medium (after removal of the cells) and of the KOH were taken at the end of the incubation period for radioactivity determinations on the residual glucose, and on the respiratory $^{14}CO_2$ evolved, respectively. There was no significant source of radioactivity in the medium after the experiment other than glucose.

The various specimens were assayed for radioactivity by the same techniques as were used in earlier work (Moses, 1957).

Substances containing ^{14}C were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Incorporation of radioactivity from labelled glucose

(a) *Unstarved cells*

Assay of the radioactivity in the various fractions obtained from the cell extracts showed that glucose was metabolized very rapidly (Table 1). The fractions contained radioactive substances from the medium as well as from the extracted cells, and the very high activity in the non-ionic fraction in the early stages was due largely to unmetabolized glucose.

Two substances appeared in the chromatograms of the cationic fraction, one of which was glutamic acid, the other not being identified. Substantially the same pattern of radioactivity was demonstrated by the cationic fraction of each of the cell extracts from 15 sec. to 6 min.

In the anionic fractions only succinate was identified, though a number of other active substances were present. Succinate appeared labelled in the 15 sec. extract, and remained visible in the 45 sec., and very faintly in the

Table 1. *Distribution of radioactivity in unstarved cells fed uniformly-labelled glucose*

A number of samples of cell suspension, each containing 60 mg. dry wt. of cells, were supplied with 12.5 μ c. (160 μ g.) of (14 C)glucose. At intervals samples were mixed with boiling ethanol to kill and extract the cells. The extracts were fractionated into anionic, cationic and non-ionic fractions by ion-exchange resins. The activity of each fraction was determined, together with the radioactivity incorporated into the insoluble cell material. The values given below are the total for each sample of suspension. As the cells were not separated from the medium before extraction, the high activity of the non-ionic fraction is largely due to unutilized glucose.

Incubation period (sec.)	Anionic fraction (counts/min.)	Cationic fraction (counts/min.)	Non-ionic fraction (counts/min.)	Insoluble material (counts/min.)
15	225,000	191,000	8,400,000	30,000
45	185,000	286,000	7,760,000	31,000
120	672,000	230,000	6,727,000	106,000
360	2,268,000	297,000	2,644,000	699,000

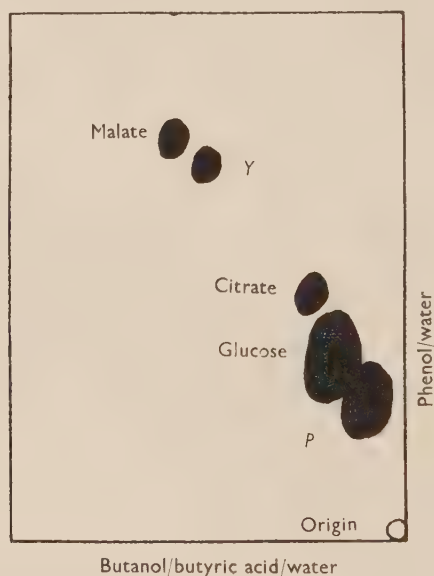


Fig. 1. Diagram of chromatogram of non-ionic fraction of cell extract showing the location of the radioactive areas mentioned in the text. Cells supplied with radioactive glucose extracted with hot ethanol. Extract separated into cationic, anionic, and non-ionic fractions with ion-exchange resins.

2 min. extracts. There was a considerable degree of change in this fraction, substances present in the 15 sec. extracts decreasing greatly in activity or being entirely absent after 6 min., and different active substances taking their places.

The 'non-ionic' fraction showed a rapidly decreasing radioactivity during the course of the experiment corresponding to the utilization of labelled glucose. After 6 min. almost the entire supply of glucose had been metabolized. Malate and citrate both incorporated activity from glucose. In 15 sec. almost every compound in this fraction which was eventually to appear containing labelled carbon could already be seen in the radioautograms. As the experiment progressed these compounds gradually disappeared as the supply of glucose was utilized. An unidentified substance *Y* appeared only in the 15 sec. extract. Malate and citrate remained labelled after 6 min. A diagram indicating the relative positions of the various substances in this fraction is shown in Fig. 1.

No activity could be found on the chromatograms in the areas known from preliminary experiments to be the locations of α -ketoglutarate, fumarate, pyruvate, malonate, tartarate, oxalate, glyoxylate, oxalacetate, *isocitrate*, *cis*-aconitate, itaconate, lactate, dihydroxyacetone, glucose-1-phosphate, glucose-6-phosphate, fructose-1:6-diphosphate, phosphoglycerate, 6-phosphogluconate, gluconate, 2-ketogluconate and 5-ketogluconate. While no attempt was made to identify these substances chemically owing to the small amounts of cell extracted, Millbank (1954) has reported the presence of low concentrations of α -ketoglutarate and pyruvate in cell extracts of this fungus, and fumarate has also been detected.

(b) Starved cells

The distribution of radioactivity in the various fractions of the extracts of starved cells is given in Table 2. Glucose again was metabolized rapidly, and a comparison with the values given in Table 1 shows that the activity present in the extract after 15 sec. was approximately similar to that present in the extract of unstarved cells after the same incubation period. However, activity was incorporated into the ethanol-insoluble material more slowly in cells which had been starved in buffer.

Table 2. *Distribution of radioactivity in starved cells fed uniformly-labelled glucose*

The details are the same as those for Table 1, except that the cells were starved for 24 hr. in 0.067 M-phosphate buffer, pH 6.8, for 24 hr. after growth.

Incubation period (sec.)	Anionic fraction (counts/min.)	Cationic fraction (counts/min.)	Non-ionic fraction (counts/min.)	Insoluble material (counts/min.)
15	211,000	402,000	6,478,000	28,000
45	249,000	446,000	6,718,000	54,000
120	216,000	434,000	6,978,000	71,000
360	283,000	543,000	6,244,000	304,000
1200	432,000	962,000	4,530,000	958,000

Radioautograms of the cationic fraction showed two weakly labelled substances which did not correspond to any amino acid tested, and were not identified.

The anionic fraction after 15 sec. contained two prominent substances, neither of which was present in the corresponding fraction from unstarved cells. One of these was identified as glucose-1-phosphate; the other was not identified. Other compounds appeared later in the experiment, among them succinate, first appearing after 2 min., and fructose-1:6-diphosphate, which appeared after 45 sec.; these substances remained visible after 20 min.

In the non-ionic fractions a pronounced glucose spot was visible throughout the incubation period and the radioactivity measured in this fraction remained correspondingly high after 20 min. (Table 2). Citrate and malate were labelled from 15 sec. onwards. There were, however, considerable differences in this fraction between the starved and unstarved cells. The unidentified substance *Y* which appeared after 15 sec. in unstarved cells did not appear at all in extracts of starved cells, nor did some other fainter spots. In the starved cells several other weak spots were visible. After 6 min. a compound appeared in starved cells (*P*) which after 20 min. was almost as intense as glucose (Fig. 1). This substance was not identified.

Incorporation of radioactivity from carboxyl-labelled acetate

The radioactivities present in the media of the cell suspensions I, II and III at the beginning and end of the incubation period are shown in Table 3, together with the activities incorporated into the cells and released in the respired carbon dioxide.

Table 3. *The utilization of acetate carboxyl carbon and release of carbon dioxide by three samples of cells*

50 ml. of each cell suspension was shaken in a 500 ml. Erlenmeyer flask in the presence of unlabelled glucose and carboxyl-labelled acetate. Measurements were made of the initial and final radioactivities in the media. In similar experiments in Warburg flasks the $^{14}\text{CO}_2$ evolved by aliquots of the cell suspensions was measured. There was no significant source of radioactivity in the medium other than acetate. The values given represent the total quantities used in each case and are expressed as counts/min. Cells of sample I were unstarved. Cells of sample II were starved overnight in phosphate buffer, and cells of sample III were starved overnight and then allowed to metabolize glucose for 3 hr.

	Sample I	Sample II	Sample III
Initial (^{14}C)acetate in the medium	26,108,000	26,108,000	26,108,000
Final (^{14}C)acetate in the medium	12,590,000	20,235,000	112,000
Thus (^{14}C)acetate utilized	13,518,000	5,873,000	25,996,000
$^{14}\text{CO}_2$ released	2,275,000	2,347,000	10,482,000
Thus ^{14}C incorporated into the cells	11,243,000	3,526,000	15,514,000

The specific activities of the glutamic acid isolated from each cell sample and of the α -carboxyl carbons are given in Table 4. From these values it is possible to calculate the percentage of the oxalacetate used for citrate formation in the tricarboxylic acid cycle which is formed from recycled C_4 -dicarboxylic acids (*K*) compared with the percentage of oxalacetate synthesized afresh from carbon dioxide and pyruvate (*P*). This *P/K* ratio is an indication of the extent of recycling taking place within the tricarboxylic acid cycle (Roberts, Cowie,

Britten, Bolton & Abelson, 1953). The results in Table 4 show that the degree of recirculation increased when the cells were starved and later resupplied with glucose.

Table 4. *Radioactivity of glutamic acid obtained from three samples of cells*

Glutamic acid was isolated from three samples of cells supplied with unlabelled glucose and carboxyl-labelled acetate. Cells of sample I were unstarved. Cells of sample II were starved for 24 hr. in phosphate buffer, and cells of sample III were starved and then allowed to metabolize glucose for 3 hr. Each glutamate sample was decarboxylated and the specific activities of the glutamic acids and their α -carboxyl carbons are given as counts/min./ μ mole. The P/K value is derived from the percentage of the oxalacetate utilized in citrate synthesis which is formed from recycled C_4 dicarboxylic acid (K) compared with the percentage synthesized *de novo* from pyruvate and carbon dioxide (P).

	Sample I	Sample II	Sample III
Specific activity of glutamic acid	4,984	9,026	15,799
Specific activity of α -carboxyl carbon of glutamic acid	1,138	2,531	4,902
P/K^*	41/59	22/78	10/90

* The P/K value for growing cells was previously found from glutamic acid labelling to be 40/60 (Moses, 1957).

Respiration of labelled glucose

By allowing similar suspensions of cells to oxidize (^{14}C)glucose and $^{14}(C_1)$ -glucose and measuring the uptake of the marked glucose and the radioactivity present in the respired carbon dioxide, it is possible, assuming that all the $^{14}CO_2$ derived from ($^{14}C_1$)glucose is released before the glucose molecule is split into smaller units, to estimate the percentage of glucose metabolized by a primary attack at the C_1 position (Heath & Koffler, 1956). The $^{14}CO_2$ produced from ($^{14}C_1$)glucose is a measure of the extent of oxidation of the C_1 atom. Knowing this, together with the total activity of the $^{14}CO_2$ from (^{14}C)glucose and the amount of (^{14}C)glucose utilized, the proportion of $^{14}CO_2$ produced from the other carbon atoms can be calculated. As a symmetrical breakdown of glucose would produce $^{14}CO_2$ from all carbon atoms, the preferential quantity derived from the C_1 position can be used to estimate the extent of primary attack at this position.

This technique has been carried out with cells in the four physiological states described above, and the results are presented in Table 5. The findings showed that while growing cells attacked about 11 % of the glucose primarily at the C_1 position, in resting cells this value had risen to nearly 20 %; after starvation the utilization of this route was less than 1 % immediately glucose was supplied, but 3–4 hr. later it had risen to 5 % (Table 5).

Under anaerobic conditions resting cells (sample I) produced about six times more $^{14}CO_2$ from (^{14}C)glucose than from ($^{14}C_1$)glucose of the same specific activity. In the Embden–Meyerhof glycolysis scheme no carbon dioxide would be produced from the C_1 of glucose; the fungus thus uses some other pathway to some extent in glucose fermentation. DeMoss (1953) has recorded an instance of carbon dioxide being produced from the first carbon atom of glucose during fermentation by *Pseudomonas lindneri*.

Table 5. *Utilization of (^{14}C)glucose and ($^{14}\text{C}_1$)glucose, and release of respired $^{14}\text{CO}_2$, by four samples of cells*

Each Warburg flask contained 2 ml. of cell suspension in 0.067M-phosphate buffer, pH 6.8 (except cells of sample IV, which were suspended in growth medium), and 0.2 ml. of N-KOH in the centre well. (^{14}C) or ($^{14}\text{C}_1$) glucose was added from the side arm to give a concentration of 0.002M. The gas phase was air for samples I-III, and O_2 for sample IV. After being shaken for 1 hr. at 25° analyses were made of the medium and KOH for the activities of the residual glucose and respired carbon dioxide, respectively. The initial activities of both the labelled substrates were adjusted to the same value. Cells of sample I were unstarved, cells of sample II were starved for 24 hr. in phosphate buffer, and cells of sample III were starved and then allowed to metabolize glucose for 3 hr. Cells of sample IV were growing. Values for the radioactivities are totals and are expressed as counts/min./flask. The method of calculation is that of Heath & Koffler (1956).

	Sample I	Sample II	Sample III	Sample IV
Initial (^{14}C)glucose or ($^{14}\text{C}_1$)-glucose in the medium	1,676,000	1,676,000	1,676,000	1,676,000
Final (^{14}C)glucose or ($^{14}\text{C}_1$)glucose in the medium	107,000	659,000	242,000	1,508,000
Thus (^{14}C)glucose or ($^{14}\text{C}_1$)glucose utilized	1,569,000	1,017,000	1,434,000	168,000
$^{14}\text{CO}_2$ produced from ($^{14}\text{C}_1$)-glucose	679,000	90,000	275,000	83,000
$^{14}\text{CO}_2$ produced from (^{14}C)glucose	427,000	84,000	219,000	68,000
Percentage of the total ($^{14}\text{C}_1$)-glucose activity utilized appearing in CO_2	43	8.8	19	49
Activity in each C atom of (^{14}C)-glucose utilized	262,000	170,000	239,000	28,000
Activity in $^{14}\text{CO}_2$ from (^{14}C)-glucose which is derived from oxidation of C_1	112,000	15,000	46,000	13,000
Thus, activity in $^{14}\text{CO}_2$ from (^{14}C)glucose derived from C_{2-6}	315,000	69,000	173,000	54,000
Total activity in C_{2-6} of (^{14}C)-glucose utilized	1,308,000	848,000	1,195,000	140,000
Percentage of activity in C_{2-6} utilized appearing in CO_2	24	8.2	14	38
Minimum proportion of glucose metabolized by a primary attack at C_1 (%)	19	0.6	5	11

DISCUSSION

Cells of this organism possess all the enzymes necessary to carry out the reactions of the tricarboxylic acid cycle (Moses, 1955c), and it has recently been shown that the cycle plays an important part in the respiration of growing cells (Moses, 1957). The present finding that radiocarbon from glucose is incorporated into succinate, malate, citrate, and glutamate in unstarved cells is in accord with the earlier results.

As many of the substances deriving radioactivity from glucose were not identified it was not possible to come to any conclusions as to the pathway of glucose metabolism save that the tricarboxylic acid cycle appeared to be involved and that, in starved cells at any rate, glucose carbon was metabolized at least to some extent via glucose-1-phosphate and fructose-1:6-diphosphate.

More interesting are the differences which were found between the starved and the unstarved cells. It was previously suggested (Moses, 1955*a*) that permeability considerations played some small part in the manifestation of the lag period of glucose metabolism when this substance was supplied to starved cells. Although the present work has shown that the rate of utilization of glucose carbon was slower in starved than in unstarved cells, the first appearance of radioactivity from glucose in a number of other substances including malate and citrate was nevertheless of the same order of rapidity in both types of cells.

Starvation resulted in a considerable change in the pattern of incorporation of glucose carbon. A number of substances, including glutamate, which became radioactive in unstarved cells possessed no detectable activity in starved cells. On the other hand, in starved cells many areas of activity appeared on the chromatograms which did not correspond with any in unstarved cells: notable among these were glucose-1-phosphate and fructose-1:6-diphosphate. It is evident that starvation resulted in a considerable metabolic rearrangement and that the pathways involved in glucose utilization were different before and after starvation.

These conclusions are further borne out by kinetic studies using labelled glucose and acetate with cells in different physiological conditions. Of the (^{14}C)glucose carbon utilized, growing cells (sample IV) released 40 % as carbon dioxide, resting cells (sample I) released 27 %, starved cells initially released 8 % (sample II) which rose later to 15 % (sample III) (Table 5). The minimum percentage of glucose attacked at the C_1 position increased when the cells were removed from the growth medium and maintained in a resting condition, decreased after starvation and tended to return towards the resting value when the cells were supplied with glucose for some hours after starvation.

The degree of recycling in the tricarboxylic acid cycle is a measure of the extent to which the cycle is used as a mechanism for synthesis (e.g. of aspartic and glutamic acids) or as a respiratory pathway (Roberts *et al.* 1953). The synthetic significance of the cycle diminished after starvation and the cycle continued to be used predominantly as a respiratory mechanism for several hours after the cells were again allowed to respire glucose.

Labelled glucose and fructose phosphate are derived from radioactive glucose in starved but not in unstarved cells, and while in starved cells the proportion of glucose attacked at the first carbon position is nearly zero, in unstarved resting cells the proportion is nearly 20 %.

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The Growth of Mixed Populations of *Chilomonas paramecium* and *Tetrahymena pyriformis*

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SUMMARY: In mixed cultures where *Chilomonas paramecium* and *Tetrahymena pyriformis* W were in competition for the same dissolved food, the population size of both species (expressed in terms of total number of organisms), as well as the size and shape of individual organisms, were all affected by the presence of the other species. The size of the *Chilomonas* population was significantly larger in mixed than in single cultures up to the stationary phase, after which the *Chilomonas* rapidly decreased in numbers and finally died out. The *Tetrahymena* population was also larger in mixed cultures in the early stages, though by the beginning of the stationary phase this was not so. Only when the *Chilomonas* had died out did the *Tetrahymena* population again reach the size of that in single cultures. In terms of total volume of organisms, the growth of the *Tetrahymena* population was not as good initially as in single cultures. Later, when the *Chilomonas* population became very small, the total volume of the *Tetrahymena* population increased and finally reached the size of the population in single cultures.

Chilomonas paramecium and *Tetrahymena pyriformis* were both more slender in mixed than in single cultures, the differences being statistically significant. Variability of the organisms was greater in mixed cultures and changed with the age of the population. During population growth, the individual size of the *Chilomonas* and the *Tetrahymena* changed; but the time-course of change was different for the two species. While the size of the *Chilomonas* increased in the logarithmic phase and then decreased, the size of the *Tetrahymena* decreased in the logarithmic phase and increased in the phase of negative growth acceleration. In old cultures, the size of both species was smaller than at the beginning of the stationary phase.

The growth of mixed populations of protozoa, competing for the same food supply, has hitherto been investigated only in organisms feeding on bacteria. In the studies of Gause (1932, 1934, 1935*a, b*), attention was concentrated on the growth of the protozoa only, while the growth of the bacteria and their influence on the outcome of competition were neglected. The starting-point of the present investigation was a search for two species of protozoa which did not prey on each other and which grew well in the same organic medium, in bacteria-free cultures, and could therefore be used to examine the interaction of competitors not related as predator to prey. Two such species are *Chilomonas paramecium* Ehrenberg and *Tetrahymena pyriformis* (Ehrenberg) Lwoff, strain W. Hall (1950) reported that *C. paramecium* was taken as food by *T. geleii* W. In the present experiments, however, neither direct observation nor the results of staining with Lugol's iodine (as a test for starch from ingested *Chilomonas*) ever suggested that strain W takes the present *Chilomonas* as food.

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METHODS

The data concerning the strain of *Chilomonas paramecium* used were given in a previous paper (Mučibabić, 1956). Corliss (1952, 1953a) described the history, systematics and morphology of *Tetrahymena pyriformis*; the strain W was isolated by C. L. Claff in 1939 (Corliss, 1952), and subcultures for the present investigation were obtained from the Culture Collection of Algae and Protozoa, Botany School, Cambridge. Fresh clones were established to ensure that the populations of the species used in these experiments were homogeneous. It was found that *T. pyriformis* W and *C. paramecium* both grew well in a solution of 0.1 % Proteose peptone (Difco) and 0.1 % sodium acetate; indeed the *Chilomonas* grew better in this than in the more usual 0.1 % beef extract (Difco) medium.

The 0.5 ml. cultures were inoculated with ten organisms of each species, and single populations of each species served as controls, the inoculum of the latter being ten individuals of the *Chilomonas* or of the *Tetrahymena*. All cultures were maintained at 22.5°. It was necessary to increase the number of observations over the initial period of growth, because the duration of the logarithmic phase varies in individual cultures. Cultures of the *Chilomonas*, for example, reached the maximum stationary phase in 5–7 days; and cultures of the *Tetrahymena* in 8–10 days.

Before starting to count, a loopful of every culture was spread over a slope of Bacto-nutrient agar (Difco), subsequently incubated, and examined for signs of bacterial contamination. It is worth mentioning that *Tetrahymena pyriformis* W grows on Bacto-nutrient agar, forming small round patches that can easily be mistaken for colonies of bacteria. The cultures were counted completely, or by sampling, and then discarded (Mučibabić, 1956).

Growth of population of Chilomonas paramecium and Tetrahymena pyriformis in terms of total numbers of organisms

Table 1 shows the growth of mixed and single populations of the *Chilomonas* and the *Tetrahymena* in terms of total numbers of organisms. The maximum size of the population of the *Chilomonas* in mixed cultures was about 170 % of the maximum population in single cultures, and growth was generally better in the mixed populations during the first 11 days. The larger size of population is statistically significant from the sixth day onwards. This large number of *Chilomonas* in mixed populations was not maintained, however; it began to decrease, slowly at first, and then rapidly, until the end of the twenty-second day. From the twelfth day the number was significantly smaller than in single cultures. Later, there was a slight increase in numbers, but after some fluctuations, the *Chilomonas* disappeared completely by about the thirty-sixth day.

The course of growth of the population of the *Tetrahymena* in mixed cultures differs from that of the *Chilomonas*. During the first 7 days its numbers were larger than in the control single cultures, and this difference was statistically significant after the third day. Later, the numbers were smaller, and from the

ninth day onwards, significantly so. At the beginning of the maximum stationary phase, the number was about 16% smaller than in control single cultures. The number did not change significantly until the sudden drop in

Table 1. Growth of single and mixed populations of *Chilomonas paramecium* and *Tetrahymena pyriformis* in terms of total number of organisms

\bar{x} = mean value; s = standard deviation; n = number of observations.

Age (days)	Single populations						Mixed populations					
	<i>C. paramecium</i>			<i>T. pyriformis</i>			<i>C. paramecium</i>			<i>T. pyriformis</i>		
	\bar{x}	s	n	\bar{x}	s	n	\bar{x}	s	n	\bar{x}	s	n
1	42.4	11.2	17	210.9	55.4	13	46.6	13.1	17	231.2	79.6	17
2	308.4	71.3	19	1,046	175	16	358.8	180	18	976	314	13
3	1,905	1,170	20	2,200	320	20	1,785	631	20	2,235	710	20
4	10,163	7,530	20	4,190	1,710	19	11,400	7,440	18	6,720	3,500	18
5	47,000	25,100	20	10,640	6,510	16	53,600	32,900	18	19,520	10,030	18
6	75,300	14,000	19	20,350	11,700	16	128,600	27,500	15	31,030	6,090	15
7	79,600	6,900	10	27,620	7,590	9	135,100	26,500	9	33,230	4,330	9
8	79,600	7,500	7	41,600	10,800	6	126,900	31,300	10	32,600	2,700	10
9	81,400	7,500	7	44,600	7,700	7	111,600	12,600	9	34,770	6,300	9
10	75,600	12,700	7	42,700	7,300	7	96,800	31,700	9	37,200	6,100	9
11	78,000	6,000	7	45,600	5,500	7	97,200	16,400	9	35,200	3,600	9
12	84,250	13,700	4	46,500	3,800	4	70,900	20,100	8	33,370	4,900	8
13	80,600	5,500	3	45,600	3,500	3	36,000	—	5	36,000	1,800	5
14	92,000	13,200	4	42,250	1,200	4	23,300	—	9	36,100	6,500	9
15	85,300	1,200	3	41,500	15,200	4	15,400	—	5	39,000	5,500	5
16	83,000	12,400	3	42,600	2,100	3	13,600	—	5	35,200	6,000	5
17	79,300	8,100	3	46,000	3,600	3	14,800	—	5	34,600	6,700	5
18	84,000	6,200	3	45,300	2,500	3	4,400	—	5	37,200	9,100	5
19	81,600	20,300	3	47,000	1,410	2	6,000	—	5	38,000	5,300	5
20	77,500	2,000	2	49,000	5,600	2	5,800	—	4	40,000	5,200	4
21	73,000	—	1	41,000	—	1	1,300	—	3	33,600	4,900	3
22	86,000	11,300	2	43,000	—	1	148	—	3	33,300	5,500	3
23	80,000	—	1	38,000	—	1	360	—	3	36,600	3,200	3
24	77,000	0	2	56,000	—	1	730	—	3	43,300	13,600	3
25	69,000	—	1	—	—	—	830	—	3	36,300	1,500	3
26	73,500	2,000	2	—	—	—	1,028	—	3	39,000	4,400	3
27	77,000	—	1	50,000	—	1	600	—	3	46,000	7,000	3
28	82,500	7,700	2	59,000	—	1	1,338	—	3	43,700	4,400	3
29	76,000	—	1	51,000	—	1	600	—	3	43,600	5,100	3
30	73,000	9,800	2	49,000	—	1	2,190	—	1	44,000	—	1
31	71,000	—	1	50,000	—	1	230	—	3	38,600	7,000	3
32	77,500	6,300	2	55,000	—	1	940	—	3	38,700	7,400	3
33	75,000	—	1	47,000	—	1	600	—	3	47,000	6,900	3
34	—	—	—	42,000	—	1	0	—	3	44,000	7,100	3
35	81,000	—	1	—	—	—	360	—	3	43,600	2,300	3
36	73,000	—	1	50,000	—	1	0	—	3	39,600	2,900	3

the population of the *Chilomonas* began; it then showed an increase, so that near the end of the experiment the size of population did not differ significantly from that of the *Tetrahymena* in control cultures.

In the single cultures, when the populations of the *Chilomonas* and the *Tetrahymena* reached the maximum stationary phase, their numbers were maintained with but small fluctuations until the end of observations (36 days).

Growth of population of Chilomonas paramecium and Tetrahymena pyriformis in terms of the total volume of organisms

Previous measurements of the size of *Tetrahymena pyriformis*, strain W, by Loefer (1952) and Corliss (1953*a*), were made on fixed organisms. Preliminary observations showed, however, that shrinkage occurred during fixation, but was least in Champy's fixative. In order to test whether reliable estimates of the volume in life could be obtained from organisms fixed with this fixative, photographs were taken, first of a living organism *T. pyriformis*, and then of

Table 2. *Growth of single and mixed populations of Chilomonas paramecium and Tetrahymena pyriformis in terms of total volume of organisms*

Age (days)	Single populations		Mixed populations	
	<i>C. paramecium</i>	<i>T. pyriformis</i>	<i>C. paramecium</i>	<i>T. pyriformis</i>
			(thousands of μ^3)	
0	12	175	12	175
2	312	12,720	535	10,780
4	19,832	92,481	22,048	103,864
6	95,255	474,766	231,223	369,971
8	101,510	1,287,894	133,753	433,547
10	131,998	728,419	113,740	565,626
12	135,306	575,252	54,664	323,989
14	127,236	450,512	20,574	327,319
16	92,130	434,520	14,090	312,963
18	81,816	487,519	4,176	289,862
20	86,955	455,651	8,462	372,760
22	84,108	488,179	157	287,012
24	95,480	536,928	958	339,082
26	63,798	478,300	1,245	380,367
28	68,145	436,442	1,545	464,444
30	71,686	427,672	2,146	448,174
32	76,570	551,760	905	446,056
34	95,706	410,550	0	356,136

the same organism after fixation in Champy's fluid and washing in Da Fano's solution (Corliss, 1953*b*). Sixteen organisms were photographed in this way (Pl. 1, fig. 1), and the size of the live and fixed organisms (length and greatest width) was measured from the photographs. From the series of differences, t was determined (Fisher, 1950; see 'Significance of mean of a small sample'). Its value (2.59) both for length and width (for 15 degrees of freedom) showed that the differences in size between living organisms and organisms fixed in Champy's fixative were statistically significant (P lies between 0.05 and 0.02). It was evident that only measurements of living organisms were of value for a quantitative study of changes in size. Accordingly, photographs of living organisms in single and mixed cultures were taken every second day throughout the growth of the population (Pl. 1, figs. 2-4). The length and maximal width of the organism in sharp focus were measured, and the average volume of individual organisms in single and in mixed populations calculated.

These data were used for the calculation of the total volume of populations

in single and mixed cultures. The total numbers of organisms (Table 1) were multiplied by the corresponding value for the volume of individual organisms at the same age (Tables 3 and 4); the results are shown in Table 2. They indicate first, that the maximum size of population (total volume or 'biomass') reached in single cultures was not maintained for as long a time as when expressed as total number of organisms (Table 1).

The growth of the *Chilomonas* populations in mixed cultures, expressed as total volume of organisms, followed the same course in time as when expressed as total number of organisms; but the growth of the *Tetrahymena* in mixed cultures in terms of total volume was different from that in terms of total number; growth was worse than in the control cultures (except for 1 day), the maximum yield being only 44% of that in the single population.

It is to be noted that the total volume of the *Tetrahymena*, either in single or mixed populations, was always greater than the total volume of the population of the *Chilomonas*. The maximum size of the single population of the *Chilomonas* was about $231 \times 10^6 \mu^3$, while that of the *Tetrahymena* was about $1288 \times 10^6 \mu^3$. These differences occurred in cultures with the same kind and quantity of nutrient medium. They imply that the degree of utilization of the nutrient medium depended on physiological characteristics of the species.

Changes in size and shape of Chilomonas paramecium and of Tetrahymena pyriformis during the growth of single and mixed populations

Values for length and width, for the ratio of length to width, as well as for the volume, of individual organisms of the *Chilomonas* and the *Tetrahymena*, during the growth of single and mixed populations, are shown in Tables 3 and 4. These list the mean value (\bar{x}) and coefficient of variation (c.v.)* of the length and width, and of their ratio; the average volume (V), and the number of observations (n). The ratio of length to width was calculated for each organism measured. The average volume of the *Tetrahymena* was calculated in the same way as the volume of the *Chilomonas*, that is, $V = \frac{4}{3} \frac{L}{2} \left(\frac{W}{2} \right)^2$ because it appeared that the form of both organisms approximates most nearly to an ellipsoid.

Tables 3 and 4 show that the size and shape of both organisms depended on the age of the population and on the presence or absence of the other species (see also Pl. 1, figs. 2-4). It is clear that the *Tetrahymena* and the *Chilomonas* differed not only (and obviously) in average size and shape, but also in the sequence of changes in size and shape during population growth. The *Chilomonas* became larger after inoculation, while the *Tetrahymena* became smaller. The increase in size of the *Chilomonas*, both in single and mixed cultures, was confined to the early stages of growth of the population. During this period, and in the logarithmic phase of growth, the size of the *Tetrahymena* in single culture decreased (Pl. 1, fig. 2a); subsequently, in the phase

* Coefficient of variation = relative standard deviation, that is, the standard deviation expressed as a percentage of the mean.

Table 3. *Changes in size and shape of Chilomonas paramecium during population growth in single and mixed cultures*L=length (μ .); W=width (μ .); V=volume (μ^3); c.v.=coefficient of variation

Age (days)	Single population								Mixed population							
	L (μ .)		W (μ .)		L/W		V (μ .) ³	n	L (μ .)		W (μ .)		L/W		V (μ .) ³	n
	\bar{x}	C.V.	\bar{x}	C.V.	\bar{x}	C.V.			\bar{x}	C.V.	\bar{x}	C.V.	\bar{x}	C.V.		
0	24.1	10.17	9.95	7.24	2.43	10.70	1247	22	24.1	10.17	9.95	7.24	2.43	10.70	1247	22
2	26.2	17.02	11.0	14.91	2.43	18.93	1659	30	24.4	12.17	10.8	7.87	2.25	8.89	1490	12
4	27.7	10.58	11.6	13.56	2.43	12.76	1951	45	26.1	10.65	11.9	13.53	2.22	15.32	1934	34
6	22.8	10.75	10.3	7.96	2.23	13.90	1265	10	27.9	9.75	11.1	11.62	2.53	11.86	1798	43
8	24.4	14.92	10.0	11.20	2.44	13.52	1276	32	23.8	14.12	9.2	10.65	2.61	15.71	1054	46
10	26.6	12.56	11.2	11.70	2.40	12.92	1746	32	22.9	11.57	9.9	13.84	2.37	17.30	1175	47
12	24.5	11.10	11.2	10.18	2.20	10.0	1606	27	20.4	11.23	8.5	13.53	2.44	12.70	771	33
14	24.9	12.57	10.3	7.09	2.44	12.70	1383	20	22.3	13.05	8.7	15.29	2.61	13.79	883	30
16	22.1	13.21	9.8	16.02	2.22	10.36	1110	51	22.9	13.54	9.3	12.37	2.48	10.48	1036	35
18	22.5	11.29	9.2	8.0	2.44	10.25	974	12	22.4	12.63	9.0	14.33	2.54	20.47	949	7
20	21.9	13.15	9.9	13.74	2.22	13.06	1122	30	26.3	9.85	10.3	9.71	2.55	8.63	1459	57
22	20.3	10.99	9.6	10.42	2.15	9.77	978	38	24.1	12.06	9.2	11.85	2.63	12.53	1067	62
24	21.5	6.84	10.5	9.14	2.06	9.22	1240	30	25.1	12.31	10.0	9.80	2.52	11.11	1313	40
26	19.2	13.54	9.3	16.77	2.08	10.58	868	20	22.7	20.13	10.1	10.79	2.24	14.73	1211	45
28	19.5	11.33	9.0	11.33	2.20	11.81	826	42	23.0	13.52	9.8	14.18	2.38	14.29	1155	34
30	20.8	10.58	9.5	12.21	2.21	10.41	982	21	22.3	11.70	9.2	10.33	2.43	13.58	980	23
32	20.5	10.05	9.6	10.10	2.16	10.65	988	21	21.3	10.52	9.3	12.04	2.32	14.22	965	20
34	23.0	11.70	10.1	8.91	2.29	12.23	1227	17								

Table 4. *Changes in size and shape of Tetrahymena pyriformis-W during population growth in single and mixed cultures*L=length (μ .); W=width (μ .); V=volume (μ^3); c.v.=coefficient of variation

Age (days)	Single population								Mixed population							
	$L(\mu.)$		$W(\mu.)$		L/W		$V(\mu.^3)$	n	$L(\mu.)$		$W(\mu.)$		L/W		$V(\mu.^3)$	n
	\bar{x}	C.V.	\bar{x}	C.V.	\bar{x}	C.V.			\bar{x}	C.V.	\bar{x}	C.V.	\bar{x}	C.V.		
0	69.1	11.14	22.0	12.77	3.17	11.36	17,491	16	69.1	11.14	22.0	12.77	3.17	11.36	17,491	16
2	42.8	9.35	23.3	11.85	1.85	8.11	12,161	22	43.6	8.81	22.0	11.86	2.0	8.50	11,045	30
4	49.3	6.25	29.2	6.94	1.69	4.75	22,060	36	48.8	9.94	24.6	11.46	1.98	7.58	15,456	39
6	56.9	12.39	28.0	18.79	2.06	11.65	23,330	15	50.3	9.45	21.3	10.89	2.35	10.21	11,923	12
8	62.4	7.07	30.8	9.81	2.04	9.31	30,959	39	54.5	8.06	21.6	16.81	2.56	13.28	13,299	35
10	54.3	10.42	24.5	16.82	2.25	12.01	17,059	23	55.4	9.46	22.9	22.49	2.36	10.59	15,205	36
12	52.1	7.35	21.3	11.78	2.46	6.10	12,371	30	47.8	9.81	19.7	8.02	2.43	7.0	9,709	58
14	46.2	8.68	21.0	6.29	2.20	7.27	10,663	24	49.1	12.89	18.7	10.70	2.63	9.13	9,067	32
16	41.8	11.0	21.6	8.29	1.94	8.25	10,200	80	42.3	8.54	20.0	9.55	2.14	8.41	8,891	40
18	43.3	13.70	21.8	19.95	1.93	18.13	10,762	44	40.0	9.45	19.3	2.12	2.07	6.76	7,792	49
20	41.8	7.99	20.9	7.99	2.01	6.97	9,549	33	42.4	10.71	20.5	5.17	2.09	9.56	9,319	51
22	48.3	13.60	21.2	14.91	2.29	11.35	11,353	16	42.9	12.68	19.6	15.61	2.20	7.73	8,619	40
24	43.2	10.16	20.6	9.76	2.10	8.57	9,588	43	40.2	11.54	19.3	9.50	2.10	9.52	7,831	56
26	43.1	8.84	20.6	8.40	2.10	8.57	9,566	41	43.1	5.85	20.8	21.44	2.10	10.95	9,753	41
28	46.6	9.27	19.7	9.24	2.37	8.02	9,458	61	49.8	12.93	20.2	21.34	2.54	18.78	10,628	38
30	40.9	7.53	20.2	11.24	2.04	8.86	8,728	23	50.7	15.48	19.6	29.80	2.70	18.89	10,186	24
32	45.2	10.82	20.6	7.28	2.19	8.68	10,032	39	49.5	19.43	21.1	32.89	2.44	16.39	11,526	53
34	43.2	11.94	20.8	6.30	2.07	9.18	9,775	25	40.7	9.43	19.5	19.60	2.01	7.46	8,094	61

of negative growth acceleration, it became larger again (Pl. 1, fig. 2*b*). This increase in size was followed by a sudden decrease, at the beginning of the stationary phase (Pl. 1, fig. 2*c*). In the mixed population, the *Tetrahymena* never reaches the maximum size of organisms in the single population (Pl. 1, fig. 4*a-d*); and later its decrease in size was greater than in control cultures. Nevertheless, the size of the *Tetrahymena* in mixed populations increased again when the *Chilomonas* was nearly suppressed (Pl. 1, fig. 4*d*).

The decrease in size of the *Chilomonas* in the phase of negative growth acceleration was greater in single than in mixed cultures. When the single population of the *Chilomonas* stopped growing, however, the size of individuals increased again (Pl. 1, fig. 3*c*), surpassing the size of organisms in mixed cultures, but did not become as large as that of organisms in the early stages of growth of the single culture. The individual size of the *Chilomonas* in mixed cultures also began to increase as soon as the density of the population became low, and surpassed that of organisms in control single cultures of approximately the same age.

The ratio of length to width has been adopted as a quantitative expression of the shape of the organisms. Generally speaking, the values of this ratio are larger in mixed populations than in single. This means that the organisms are more slender when grown in mixed cultures than in single cultures; and these differences in shape are more accentuated in the *Tetrahymena* than in the *Chilomonas*.

Tetrahymena pyriformis W was plump in the logarithmic phase of growth; but in the phase of negative growth acceleration, and in the first part of the stationary phase, the organisms were slender. In older cultures, both slender and plump organisms were present. *Chilomonas paramecium*, however, was plump only in single cultures more than 20 days old. In mixed cultures it was more slender, except in the logarithmic phase of growth when the organisms were plumper than in single cultures.

Tetrahymena pyriformis W swam more rapidly in the mixed cultures than in single culture. This greater speed of swimming was not observed at the beginning of population growth, but was very noticeable from the phase of negative growth acceleration onwards. In old cultures, however, it was not observed. From their stream-lined shape in cultures 8 days old (Pl. 1, fig. 2*b*) one might indeed expect that organisms from the mixed populations would be faster swimmers than those grown in single culture.

Tables 3 and 4 show that, on the whole, the coefficients of variation of length, width and of their ratio, are larger in mixed populations. It is interesting that variation in the shape of the *Chilomonas* was more accentuated than variation in size; while for the *Tetrahymena* the converse was true.

Variation of the *Chilomonas* in single population was greater only at the beginning of the maximum stationary phase; while in a mixed population high values of the coefficients appeared almost throughout population growth.

Increased variation of the *Tetrahymena* in single cultures was exhibited at the end of the phase of negative growth acceleration, and at the beginning and in the middle of the maximum stationary phase. In mixed cultures,

however, the first increase in the coefficients of variation occurs at the beginning of the maximum stationary phase. The values of the coefficients of variation for the *Tetrahymena* were maximal when the *Chilomonas* was almost completely suppressed, and the population of the *Tetrahymena* had begun to increase. How great is the variation in size and shape of the *Tetrahymena* in mixed cultures at this time can be seen from Pl. 1, fig. 4d.

The values of length, width, and the ratio of length to width of the *Chilomonas* and the *Tetrahymena*, in single and mixed cultures, were compared statistically, using the *t*-test of significance. The results showed that differences of means of length and width of organisms in single and mixed populations were significant only from the fourth to the sixth day after inoculation. On the other hand, the difference in shape of the *Tetrahymena* from single and mixed cultures is significant from the beginning of measurements; it was almost significant for the *Chilomonas* also. As might be expected, the significance of means changes during population growth; but on the whole the differences between the organisms from single and mixed cultures are significant.

The maximal and minimal values of length, width and their ratio in single and mixed populations were also statistically compared. They were all highly significant ($P < 0.001$).

DISCUSSION

These experiments have shown that the growth of two protozoan species in mixed populations is not as simple a matter as certain mathematical theories have suggested. Statistically significant differences in the population size of *Chilomonas paramecium* and *Tetrahymena pyriformis* W, between single and mixed cultures, appeared at the beginning of population growth, and implied better growth of both species in mixed populations. Later, however, there was significantly better growth of both species in single populations. Now *C. paramecium*, a species of which the maximum population was nearly twice as large in mixed as in single cultures, was suppressed by *T. pyriformis* W, of which the maximal population was larger in single than in mixed cultures. According to theories of competition between two species, however, one would not expect suppression of the species that grows better in mixed cultures from the logarithmic to the stationary phase.

The reason for the better initial growth of both species in mixed cultures is unknown. Obvious possibilities are: that certain products of the metabolism of one species favoured population growth of the other; or that one species removed something from the medium and so made it more suitable for the growth of the other species. Whatever its nature, this conditioning of the medium took a few days, since significantly greater numbers of the *Tetrahymena* in mixed cultures only appeared on the fourth day, and those of the *Chilomonas* only on the sixth day after inoculation. The greater concentration of carbon dioxide in mixed cultures (due to a larger number of organisms) might conceivably stimulate growth of the *Chilomonas* population (Jahn, 1936; Pace & Ireland, 1945).

The growth rate (Mučibabić, 1956) of the population of the *Tetrahymena*, both in single and mixed cultures, was greater than that of the *Chilomonas* population during the first day; but later, the growth rate of the *Chilomonas* was constantly greater. From its growth rate, as well as from the maximum population size, one might expect the *Chilomonas* to win in competition with the *Tetrahymena*; but the *Chilomonas* was suppressed.

On general grounds, Huxley (1942) emphasized that change in environmental conditions may affect the results of competition, and Hutchinson (1941) showed how a successional series in any system may be caused by internal or external changes modifying properties which bear directly on the struggle for existence. Crombie (1945) also claimed that the mathematical theories of competition between two species hold only so long as the conditions of experiment remain constant; and it seems evident that in cultures such as those examined here, there must be a steady change of environmental conditions throughout the life of the culture.

Salt (1932) showed for a very different organism, namely the dipteran *Lucilia sericata*, that the population growth assumed a different appearance according to whether population size was expressed as number of organisms or as weight of organisms. This result is analogous to the observed difference in the effects of competition between the *Chilomonas* and the *Tetrahymena* used here, when the size of the population is expressed as numbers or volume of organisms.

McVeigh & Brown (1954) compared the growth of the flagellates *Chlamydomonas chlamydogama* and *Haematococcus pluvialis*, in single and mixed population, when the cultures were 2 and 3 weeks old. They found better growth in single cultures after 2 weeks, and in mixed cultures after 3 weeks. Since they did not follow the growth of the population throughout, their results are not fully comparable with the findings of the present experiments, though to some extent they confirm the observed better growth of the *Chilomonas* and the *Tetrahymena* in a mixed population at a particular age of the culture.

The observation that the size of the *Tetrahymena* and *Chilomonas* organisms in mixed culture was significantly different from the size of organisms in single culture, and that there were marked differences between the sizes of organisms of the same culture at different ages of the population, implies that one cannot with safety measure a certain number of organisms from a single culture and use the mean values obtained from such measurements for calculating the size of population, in terms of total volume of organisms, for cultures of different age and composition, as did Gause (1935*a, b*) in his experiments on competition between two species of ciliates.

The data on variability suggest that the conditions of competition between two species induce an increased variability in size and shape of organisms in mixed cultures. This was particularly striking in the *Tetrahymena* at the time when the *Chilomonas* was nearly suppressed. It implies that changes occurred both in volume and in surface area of the organisms; in the *Tetrahymena*, either the kinetics increased in length, or the distance between them increased during an increase in surface area, or both may have happened simultaneously. These are matters for future investigation.

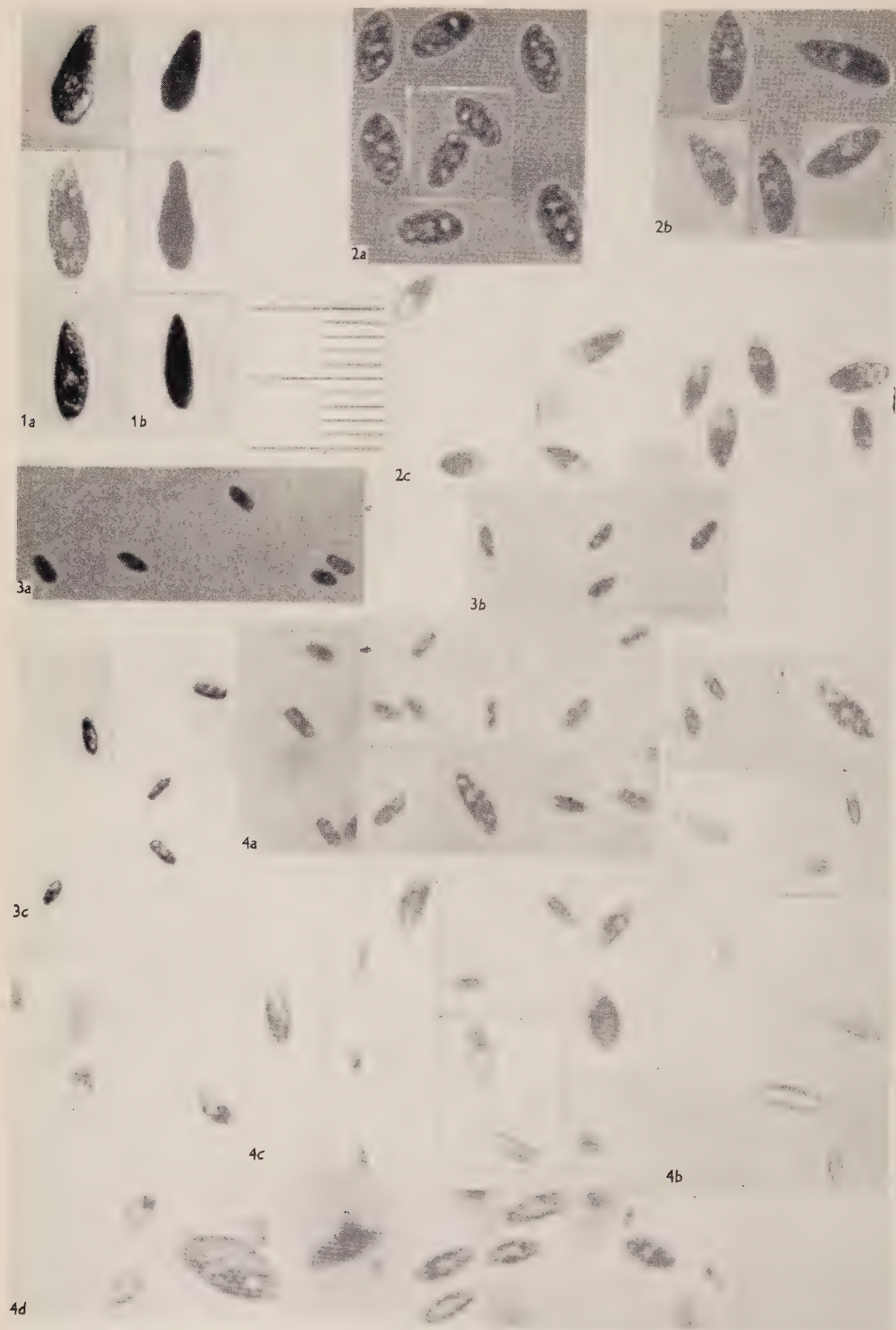
Ormsbee (1942) noted that there was variability in size of *Tetrahymena pyriformis* W in the stationary phase of population growth; but he judged changes in size of organisms from changes in length only, and claimed that the ratio of length to width did not vary significantly during population growth. The present experiments have shown, however, that profound changes in the ratio of length to width occurred in the logarithmic phase of population growth of *T. pyriformis* W. Ormsbee also observed (as here described, p. 565) a decrease in size of the *Tetrahymena* during the logarithmic phase of growth and a subsequent increase in size in the stationary phase; in the present experiments, however, an increase in size was observed in the phase of negative growth acceleration. In contradiction to my results and to those of Ormsbee, however, Slater & Elliott (1951) found that the volume of individual organisms of *T. geleii* was maximal at the end of the logarithmic phase. It was claimed by Loefer (1952) that all strains of *T. geleii* reached full size after 48 hr.; in my experiments, however, full size was not reached until 8 days after inoculation. Loefer, however, used a medium differing from mine in kind and quantity, and maintained his cultures at a higher temperature.

The observed decrease in size of organisms in old cultures, though the total numbers remained constant, may be connected with progressive deficiency in the food available, for Weis (1954) also observed decrease in size in *Tetrahymena pyriformis* in starving cultures. Harding (1937) measured the size of *Glaucoma pyriformis* (*Tetrahymena pyriformis*) in cultures without food, and also found that size decreased rapidly at the beginning of starvation, while the organisms were still multiplying.

I wish to express my gratitude to the heads of the Botany School and of the Department of Zoology, Cambridge University, for research facilities, and to the many assistants who have spared no trouble in helping me. My sincere thanks are due to Professor E. G. Pringsheim, Dr G. Salt, Professor S. Stanković, Professor G. E. Briggs and Mr E. A. George, for stimulating discussions and useful suggestions in relation to my work. In particular I am indebted to Dr L. E. R. Picken for constant help and encouragement.

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EXPLANATION OF PLATE

Chilomonas paramecium and *Tetrahymena pyriformis* W photographed alive (except for Fig. 1b) without a coverslip in single and mixed cultures. The smaller divisions on the scale are 10 μ .

Fig. 1. *T. pyriformis*: (a) three living individuals; (b) the same three individuals fixed in Champy's fluid.

Fig. 2. *T. pyriformis* in single bacteria-free culture. (a) 4 days old; (b) 8 days old; (c) 14 days old.

Fig. 3. *C. paramecium* in single bacteria-free culture. (a) 6 days old; (b) 8 days old; (c) 14 days old.

Fig. 4. Mixed bacteria-free cultures of *C. paramecium* and *T. pyriformis*. (a) 6 days old; (b) 8 days old; (c) 14 days old; (d) 32 days old.

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The Production of Gram-positive Variants by *Azotobacter chroococcum*

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SUMMARY: Cultures of *Azotobacter chroococcum* isolated by four independent operators regularly produced Gram-positive forms, from which typical cultures of *A. chroococcum* were recovered under controlled conditions. This observation appears to confirm those of earlier workers, and is in accordance with the theory that the aerobic and anaerobic nitrogen-fixing bacteria may be closely related.

Attention has already been drawn to a number of morphological and cytochemical characters of *Azotobacter chroococcum* which suggest a relationship with the family Bacillaceae (Bisset, 1955; Hale & Bisset, 1956). This may be regarded as a reasonable conclusion, in respect of bacterial systematics, since the ability to fix nitrogen is widespread in the genus *Clostridium* of this family (Rosenblum & Wilson, 1949), and since *Rhizobium* has also been shown to give evidence of its relationship with the same group by the production of Bacillus-like variants (Bisset, 1952). In fact, the production of Bacillus-like variants of *Azotobacter* spp. has also been claimed (Löhnis & Smith, 1916, 1923); but whereas the true identity of the Gram-positive sporing strains of *Rhizobium* has been proved by their ability to produce a characteristic symbiotic infection in the appropriate host plant, no such method of authentication of the Gram-positive sporing strains of *Azotobacter* is applicable. The validity of Löhnis & Smith's conclusions has been the subject of much dispute. Nevertheless, the existence of heat-resistant spores ('cysts') and a tendency to Gram-positivity in *Azotobacter* spp. has been repeatedly reported (see review by Jensen, 1954). In the same review Jensen notes that 'the morphology of *Azotobacter*... is remarkably variable'. It is our experience that these concepts are very far from being generally accepted; the definitions of the genus given in, for example, *Bergey's Manual* (6th ed., 1948) and *Topley and Wilson's Principles* (4th ed., 1955) explicitly deny them.

The earlier critics of Löhnis & Smith are typified by Jones (1920), who spent much effort in an attempt to separate the typical and Gram-positive strains, which he admitted to be exceedingly difficult, and in which he never unequivocally claimed to have been successful. Nevertheless, Jones stated that these Gram-positive bacteria were 'species other than *Azotobacter*', but neither identified them further nor stated his evidence. Jones's observations, in fact, supported Löhnis & Smith's conclusions rather than his own. Unfortunately Löhnis & Smith provided additional ammunition for their opponents by using their observations to support a general theory of bacterial mutability. In consequence, Winogradsky (1938) ridiculed their conclusions, writing, 'Il a

donc été inattendu de voir cette théorie prise au sérieux', before proceeding to review the work of numerous observers by whom the observations of Löhnis & Smith had apparently been confirmed.

In this laboratory, what appeared to be Gram-positive variants of *Azotobacter chroococcum* have repeatedly been observed, but in view of the difficulty of proving the point have not hitherto been considered suitable material for researches upon the behaviour of the species. However, recent evidence (Bisset, 1955; Hale & Bisset, 1956) suggestive of *Bacillus*-like characteristics even in typical Gram-negative strains of *A. chroococcum* indicated the desirability of re-examining this aspect of the work of the earlier investigators. In the present study, the occurrence of Gram-positive forms in isolates of *A. chroococcum* obtained at different times by different operators has been examined, and experimental evidence is adduced to support the view that they are variants of authentic *A. chroococcum*.

METHODS

Strains of *Azotobacter chroococcum* were isolated either directly upon soil plates or upon the medium described by Bisset & Hale (1953) which contained metallic salts with mannitol as a source of energy, and no source of nitrogen. For purposes of isolation this medium was employed in fluid form except by A.C.B.-P. who used it solidified with agar. In the experience of the writers, only nitrogen-fixing bacteria will grow on this medium in either solid or liquid form. After isolation upon solid media the strains of *A. chroococcum* were grown in the liquid medium, as a further means of identification, in order to check their production of the characteristic pellicle growth. Cultures were maintained upon solid or liquid mannitol + salts medium, or upon the potato-meal agar described by Bisset (1955).

RESULTS

Forty-four isolates of organisms which possessed the characters of *Azotobacter chroococcum* were isolated during a period of 2 years by four operators, each of whom worked independently, occupying different laboratories and preparing their media separately. Of these 44 cultures, 16 produced Gram-positive forms, under substantially similar circumstances (Table 1). Because the examination of these Gram-positive forms was not the original purpose of

Table 1. *Isolation of Azotobacter strains*

Operator	Date	No. of isolates	No. of isolates from which Gram + forms were obtained
J. C. L.	June-Oct. 1954	10	5
M. H. J. (i)	July 1955	7	2
A. C. B.-P.	Oct. 1955	7	4
C. M. F. H.	Mar. 1956	5	1
M. H. J. (ii)	June 1956	15	4
Totals		44	16

these isolations in any case, the first isolates were chosen for their typical *Azotobacter* appearance. They were subcultured upon the solid mannitol-salts medium and isolated colonies were repeatedly picked, usually upon three consecutive occasions, although this differed to some extent between operators.

On repeated subculture of such isolates it was found that occasional colonies when examined microscopically consisted of bacteria which were less globular and more bacillary than the typical forms. Some, but not all of these, were slightly Gram-positive on mannitol + salts medium, whether in solid or liquid form, and markedly so after two or three subcultures upon potato-meal agar. Their colonies and cultural growth, both upon potato-meal agar and upon the mannitol + salts medium, were macroscopically indistinguishable from those of the original isolates. They resembled the Gram-positive *Azotobacter* described by Löhnis & Smith (1916, 1923), having the general appearance of the larger types of *Bacillus* (e.g. *B. cereus*). Like other strains of *Azotobacter chroococcum* they were sluggishly motile, and possessed a voluminous soft capsule which gave the characteristic appearance to the colonies. This capsular slime was also typical of the species in that it gradually turned brown with age, and was much more profuse upon the carbohydrate media already described than upon meat-infusion agar.

These Gram-positive isolates retained their characters very well upon potato-meal agar, and were maintained for periods ranging from a month to more than a year, being repeatedly plated and purified by picking separate colonies. Of these, four strains, isolated at different times by three different operators (A.C.B.-P., C.M.F.H. and M.H.J. (ii); Table 1), were chosen. All of these had been re-isolated from forms which had survived heat-treatment for 15 min. at 80° according to the techniques described by Bisset (1955). Two of the (A.C.B.-P.) isolates had further survived 100° for 2-3 min. (Bisset, 1955). As in the case of *Rhizobium* spores (Bisset, 1952) these heat-surviving cultures grew only after a lag period of several days.

These strains were subcultured at intervals of 48 hr. upon the mannitol + salts agar medium, upon which they grew freely. Colonies were picked and examined, and the various isolates subcultured upon the same medium. Under these conditions their morphology and Gram-reaction were more variable than upon the potato-meal medium, and by progressive selection of the less Gram-positive strains it was possible to obtain in each case, within a period of from 8 to 10 days, cultures which strongly resembled typical *Azotobacter* sp. These, when subcultured upon the mannitol + salts fluid medium, proved capable, after two or three subcultures at rather longer intervals because of their slower growth, of producing typical pellicle growth consisting of the highly characteristic Gram-negative almost spherical organisms. The forms derived from all four strains were alike, and differed from the original, typical *A. chroococcum*-like isolates, from which the Gram-positive forms had themselves been derived, in being *c.* 25% smaller in cell size and possessing a less voluminous capsule. Such a decrease in size is, in any case, commonplace in *Azotobacter* strains which have been maintained for long periods in artificial cultures.

DISCUSSION

The isolation, by four independent operators, of Gram-positive forms from typical cultures of *Azotobacter chroococcum* is in accordance with the findings of Löhnis & Smith (1916, 1923). These forms closely resembled sporing bacilli, but were capable of growing, in repeated subculture, in a medium which contained no source of nitrogen and would not support the growth of non-nitrogen-fixing bacteria. From such strains after prolonged culture and replating, typical *Azotobacter* could be recovered. This is also in accordance with the findings of Löhnis & Smith, and appears to us to provide adequate proof that the Gram-positive forms are in fact variants of *Azotobacter* spp. At the same time this observation provides corroboration of those recorded in the introduction to this paper, which suggest that the aerobic and anaerobic nitrogen-fixing bacteria may all be relatively closely related members of the family Bacillaceae. This conclusion has in the past been obscured by their extreme variability, and by the readiness of bacteriologists to dismiss as a contaminant anything with the characters of a sporing bacillus appearing in artificial culture.

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Pyocyanine Biosynthesis by *Pseudomonas aeruginosa*

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SUMMARY: The formation of pyocyanine by resting *Pseudomonas aeruginosa* was studied. Glutamic acid was found to be a good substrate for this synthesis and could be replaced by several related amino acids (γ -aminobutyric acid, pyrrolidone-carboxylic acid, glutamine, aspartic acid, proline, hydroxyproline, arginine, histidine, alanine), and by some organic acids active in the tricarboxylic acid cycle (succinate, fumarate, pyruvate) when added together with ammonium ions. Magnesium ions were essential for pyocyanine synthesis and could not be replaced by other metals tested (Co, Fe). Pyocyanine formation was inhibited specifically by respiratory poisons (KCN, NaN_3); NaF, on the other hand, was not inhibitory.

Pyocyanine, the blue pigment produced by *Pseudomonas aeruginosa*, is known to possess antibiotic activity especially against the Gram-positive bacteria. Chemically, pyocyanine belongs to the phenazine group and is similar in structure to iodinine, a pigment produced by *Chromobacterium iodinum* (Clemo & Daglish, 1950), and to chlororaphin, a green pigment formed by *Pseudomonas chlororaphis* (Kögl & Postowsky, 1930). The synthesis of phenazine derivatives is, therefore, not uncommon among strictly aerobic micro-organisms.

Investigations published hitherto, including those reviewed in Topley and Wilson's *Principles* (1955), and those of Young (1947), Burton, Eagles & Campbell (1947), Burton, Campbell & Eagles (1948), and Hellinger (1951) deal with pyocyanine formation in growing cultures of *Pseudomonas aeruginosa*. The object of this investigation was to study pyocyanine formation in stationary cultures, and to determine the factors essential for its formation. A preliminary note was published previously (Halpern & Grossowicz, 1954).

METHODS

The organisms used in this work were 2 strains of *Pseudomonas aeruginosa* from the stock culture collection of this Department. The bacteria were grown in Roux bottles containing 120-150 ml. Difco Nutrient Agar and incubated at 37° for 24 hr. The organisms were taken up in saline, washed several times by centrifugation and resuspended in phosphate, borate, or 'Tris' (trihydroxymethylaminomethane) buffers. The concentration of organisms was determined by measuring the turbidity of a suitably diluted suspension in a Coleman Junior Spectrophotometer.

Pyocyanine assay. The experiments on the formation of pyocyanine were carried out in unstoppered 12 x 75 mm. or 24 x 150 mm. test tubes containing the bacterial suspensions and appropriate substrates in a volume of 1 ml.; the pH value was adjusted to 7.8. The tubes were incubated statically at 37°. At

the end of the experiment the tubes were shaken vigorously by hand for about 2 min. in order to convert all the pigment to the oxidized (blue) state. The pyocyanine was then extracted from the reaction mixture with 2 ml. of chloroform and measured in the spectrophotometer at a wavelength of 675 m μ . A solution of crystalline pyocyanine 2 HCl (Nutritional Biochemicals Corp., Cleveland/Ohio, U.S.A.) made alkaline (pH 7.8) and similarly extracted with chloroform was used as a standard.

RESULTS

Amino acid requirements

Various amino acids were tested as possible substrates for pyocyanine synthesis. With glutamic acid as the sole carbon and nitrogen source, the yield of pigment was very good (100–250 μ g./ml.). However, even under optimal conditions not more than 2% of the glutamic acid was converted into pyocyanine, i.e. addition of 12 mg. glutamic acid to the system yielded 0.25 mg. of pyocyanine. This yield of pyocyanine was arbitrarily chosen as 100% to serve as a standard for the comparison of the amount of pyocyanine formed in the presence of other substrates. Pigment formation occurred also in the presence of alanine, arginine, aspartic acid, glutamine, histidine, hydroxyproline, proline, serine or threonine. No pyocyanine was formed in the presence of glycine, lysine, methionine, phenylalanine, tyrosine, tryptophane or valine. Asparagine, cysteine or leucine were adequate substrates for pyocyanine formation by only one of the two strains used (Table 1).

Since glutamic acid was found to be the best substrate for pyocyanine synthesis, compounds related to this amino acid were tested. Table 1 shows that pyocyanine formation in the presence of γ -aminobutyric acid was as great or slightly greater than in the presence of glutamic acid, while the yield of pigment in the presence of glutamine, proline, hydroxyproline or pyrrolidone-carboxylic (5-oxo-2-pyrrolidine carboxylic) acid was somewhat less. Of other amino acids known to be related metabolically to glutamic acid, ornithine proved better than arginine and citrulline. No pyocyanine was produced in the presence of lysine, but the decarboxylation product of lysine, cadaverine, produced 40% of the amount of pyocyanine which was formed in the presence of glutamic acid. The pyocyanine yield in the presence of aspartic acid was lower (20%) than that found in the presence of its decarboxylation products α - and β -alanine, which both yielded about 75%. Of the various aminobutyric acids only γ -aminobutyric acid was found to serve as an efficient substrate for pyocyanine synthesis (Table 1).

Carbon sources for pyocyanine formation

Succinate, fumarate, pyruvate and other organic acids were tested as sole sources of carbon in the presence of NH_4Cl as nitrogen source. Pyocyanine was formed in the presence of some of these substances, while others were inactive (Table 1). No pyocyanine was formed when glucose was used as the only source of carbon. Addition of malate inhibited pyocyanine production in presence of glutamate, succinate + NH_4^+ or fumarate + NH_4^+ .

Table 1. *Effect of various substrates on the formation of pyocyanine*

Total volume, 1 ml.; organisms equiv. 6.7 mg. dry wt./ml. Final concentration of substrates added: 0.1 M; MgSO_4 , 0.02 M; phosphate buffer, pH 7.8, 0.02 M. Incubation at 37° for 24 hr.

Substrate	Pyocyanine formation %*	Substrate	Pyocyanine formation %*
L-Glutamic acid	100	DL-Tyrosine	5
γ -Aminobutyric acid	110	Anthranilic acid	0
Pyrrolidonecarboxylic acid	75	α -Aminobutyric acid	5
L-Glutamine	70	α -Aminoisobutyric acid	5
DL-Proline	80	DL- β -Aminobutyric acid	15
L-Hydroxyproline	90	2,4-Diaminobutyric acid	0
DL-Ornithine	55	Cadaverine	40
DL-Citrulline	20	Betaine	10
L-Arginine	40	Tyramine	5
DL-Aspartic acid	20	Tryptamine	5
L-Asparagine†	100	Ethylamine	0
L-Alanine	85	Triethylamine	0
β -Alanine	70	Tri-aminopropanol	0
Glycine	0	Ethanolamine	0
DL-Serine	55	α -Ketoglutaric acid‡	55
DL-Threonine	35	Succinic acid‡	30
DL-Isoleucine	0	Fumaric acid‡	25
L-Leucine†	30	Pyruvic acid‡	10
DL-Valine	5	Acetic acid‡	35
DL-Methionine	0	Citric acid‡	10
L-Cysteine†	20	Malic acid‡	0
L-Cystine	0	Maleic acid‡	10
DL-Lysine	0	Malonic acid‡	25
DL-Histidine	40	Oxalic acid‡	0
L-Tryptophane	0	Glutaric acid‡	0
DL-Phenylalanine	0	Pimelic acid‡	5

* Pyocyanine formation from glutamic acid was chosen arbitrarily as 100 %. The actual amount of pigment formed was about 0.25 mg.

† Active only with one strain.

‡ NH_4Cl (0.04 M) was added.

Mineral salt requirements

Examination of the role of metals in the pyocyanine synthesis was carried out with the aid of the chelating agent ethylenediamine tetra-acetic acid (EDTA). The addition of 0.3 % EDTA prevented the formation of pyocyanine with glutamic acid as the substrate (Table 2). However, pyocyanine formation inhibited by 0.2 % EDTA was completely restored by the addition of Mg^{++}

Table 2. *The effect of MgSO_4 on formation of pyocyanine in the presence and absence of ethylenediamine tetra-acetate (EDTA)*

Organisms equiv. 6.8 mg. dry wt./ml.; glutamic acid, 0.06 M; phosphate buffer, pH 7.8, 0.02 M. Incubation at 37° for 24 hr.

EDTA (0.3 % w/v)	—	—	+	+
MgSO_4 (0.01 M)	—	+	—	+
Pyocyanine formed ($\mu\text{g./ml.}$)	144	185	8	227

ions (0.002–0.01 M) (Fig. 1). On the other hand, $\text{Co}(\text{NO}_3)_2$ and FeCl_2 (at concentrations of 0.0002 M) inhibited pyocyanine formation (Table 3). Phosphate was not necessary for pigment synthesis in the resting system. In growing cultures phosphate seems also to be dispensable (Jordan, 1899, as quoted from *Topley & Wilson*, 1955); however, the newer work of *Burton et al.* (1948) claims the essentiality of phosphate for pyocyanine formation.

Table 3. *The influence of various cations on pyocyanine production*

Organisms equiv. 4.3 mg. dry wt./ml.; phosphate buffer, pH 7.8, 0.02 M; total volume, 1 ml. Incubation at 37° for 24 hr.

	1	2	3	4	5	6	7	8	9	10	11	12
FeCl_3 (0.0002 M)	—	+	—	—	—	+	+	+	—	—	—	—
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.0002 M)	—	—	+	—	—	—	—	+	+	—	—	—
MnCl_2 (0.0002 M)	—	—	—	+	—	—	+	—	—	+	+	—
MgSO_4 (0.05 M)	—	—	—	—	+	+	—	—	+	—	+	—
Glutamic acid (0.02 M)	+	+	+	+	+	+	+	+	+	+	+	—
Pyocyanine formed ($\mu\text{g.}/\text{ml.}$)	59	10	24	64	69	21	26	6	24	25	73	10

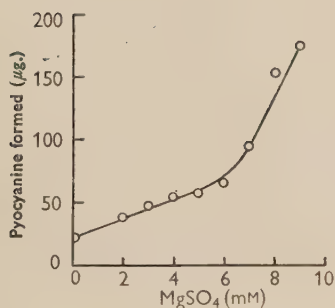


Fig. 1

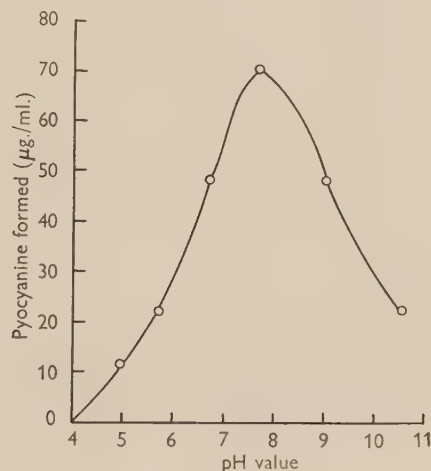


Fig. 2

Fig. 1. Dependence of pyocyanine synthesis on the concentration of MgSO_4 in the presence of ethylenediamine tetra-acetate (EDTA). Total volume, 1 ml.; glutamic acid, 0.06 M; EDTA 0.2 % (w/v); organisms equivalent to 6.5 mg. dry wt. bacteria; phosphate buffer, pH 7.8, 0.02 M; incubation at 37° for 24 hr.

Fig. 2. Effect of pH value on pyocyanine formation. Total volume, 3 ml.; glutamic acid, 0.067 M; MgSO_4 , 0.01 M; organisms equivalent to 6.5 mg. dry wt. bacteria/ml.; buffers: pH 7.2 and below, McIlvaine's buffer; above pH 7.2, 'Tris' buffer.

Effect of pH value on pyocyanine formation

Fig. 2 shows that the synthesis of pyocyanine took place over a wide range of pH values with optimum synthesis at pH 7.0–8.0. At pH values of 6.0 and 10.0 pyocyanine formation was only about 30 % of that found at pH 7–8.

The rate of pigment formation

The synthesis of pyocyanine was rather slow; the minimal time required for measurable amounts of pigment to be formed was *c.* 6–8 hr. (Fig. 3). Experiments were carried out with the aim of enhancing the rate of synthesis of pyocyanine. Since oxygen is essential for its formation, the effect of aeration on the incubation mixture was investigated. When wider test tubes (24 × 150 mm.) were used instead of the smaller ones, the final yield of pyocyanine was doubled, but the rate of synthesis was unchanged. Similar results were obtained when the reaction vessels were shaken in a Ross-

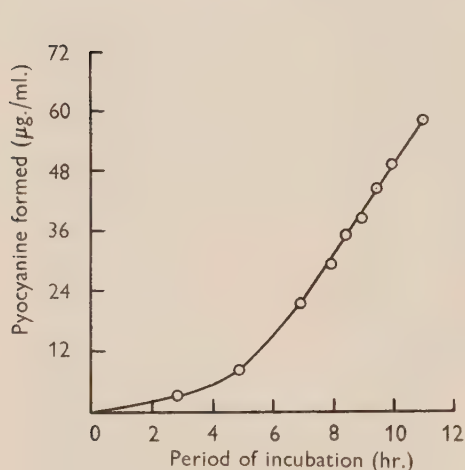


Fig. 3

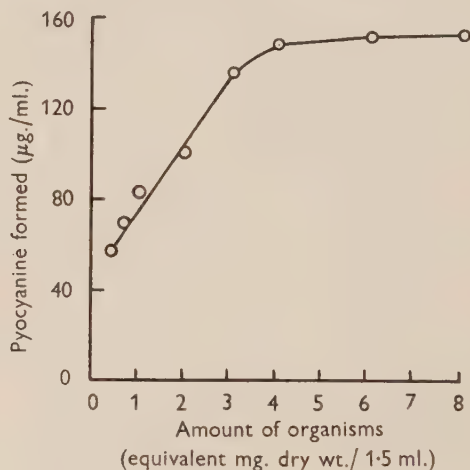


Fig. 4

Fig. 3. Pyocyanine synthesis as a function of time. Total volume: 10 ml.; glutamic acid, 0.1M; MgSO_4 , 0.02M; organisms equivalent to 7 mg. dry wt. bacteria/ml.; phosphate buffer, pH 7.8, 0.02M; incubation at 37°.

Fig. 4. Dependence of pyocyanine synthesis on the concentration of organisms. Total volume: 1.5 ml.; glutamic acid, 0.067M; MgSO_4 , 0.01M; phosphate buffer, pH 7.8, 0.02M; incubation at 37° for 24 hr.

Kershaw shaking apparatus. Stronger shaking in a reciprocating type of shaker inhibited pigment formation; in fact no pyocyanine at all was formed on vigorous shaking.

In view of the possibility that the slow synthesis was due to the lack, or insufficient formation, of some essential co-factor, the reaction mixture was enriched with yeast extract (0.2%, w/v), tomato juice (3%, v/v), or a mixture of vitamins. The vitamin mixture contained the following substances (µg./ml.): thiamine, 2.0; riboflavin, 1.0; nicotinamide, 15; Ca-pantothenate, 12.0; biotin, 0.2; pyridoxal-HCl, 2.0; pyridoxamine 2HCl, 2.0; choline, 3.0; inositol, 150.0; folic acid, 2.5; vitamin B_{12} , 0.01; *p*-aminobenzoic acid, 0.1. This was added in 0.1 ml. volumes to the 1 ml. incubation mixture.

In order to exclude the possibility that pyocyanine was synthesized by the bacteria multiplying during the incubation of the system, the following experi-

ment was designed. A series of reaction mixtures, containing a constant amount of glutamic acid, MgSO_4 and varying amounts of bacteria was set up. Fig. 4 shows that pyocyanine formation was proportional to the quantity of organisms added, indicating that the synthesis was not influenced by bacterial growth. In another experiment the concentration of organisms was kept constant while the concentration of glutamic acid varied from 0.15 to 2.4% (w/v) (Fig. 5). The results obtained were essentially similar to those presented in Fig. 4. In other experiments colony counts and turbidity measurements were made; no evidence for bacterial growth during incubation for pyocyanine formation was found. On the contrary, a considerable decrease in colony count and some decrease in turbidity was noted.

Action of various metabolic inhibitors

It was hoped that by addition of metabolic inhibitors further information about the biosynthetic pathways in pyocyanine synthesis might be obtained. Several agents were also tested with a view to securing bacteriostatic conditions for the system studied without impairing pyocyanine formation. Penicillin, aureomycin or chloromycetin did not selectively inhibit bacterial growth or pyocyanine formation. At the lower concentrations of the antibiotics used both processes continued at normal rates, whereas higher concentrations, when inhibitory, affected both the growth of *Pseudomonas aeruginosa* and pyocyanine synthesis to the same extent. KCN or NaN_3 at 0.001M inhibited pyocyanine synthesis by about 60%, and at 0.01M the inhibition of synthesis was complete. Iodoacetate (0.01M) also prevented the formation of pyocyanine. At the given concentrations cyanide and azide inhibited growth as well, whereas iodoacetate did not. Thus iodoacetate inhibited selectively pyocyanine formation. NaF was not inhibitory of growth or synthesis even at 0.01M (Table 4). Thiomersalate at a concentration of 1:30,000 completely prevented pyocyanine formation. Cysteine, glutathione and thioglycollic (mercaptoacetic) acid produced a marked decrease in the rate of pyocyanine synthesis.

Table 4. *Effect of KCN, NaN_3 , and NaF on pyocyanine synthesis*

Glutamic acid, 0.081M; MgSO_4 , 0.01M; organisms equiv. 4.3 mg. dry wt./ml.; phosphate buffer, pH 7.8, 0.02M; total volume, 1 ml. Incubation at 37° for 24 hr.

	Pyocyanine formed ($\mu\text{g./ml.}$)	Inhibition of pyocyanine formation (%)
Control (no inhibitor added)	54	0
NaF (0.01M)	53	0
KCN (0.0001M)	48	11
KCN (0.001M)	24	56
KCN (0.01M)	0	100
NaN_3 (0.0001M)	45	17
NaN_3 (0.001M)	23	57
NaN_3 (0.01M)	0	100
Mono-iodo-acetate (0.01M)	0	100

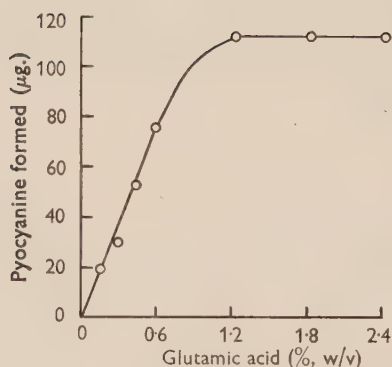


Fig. 5

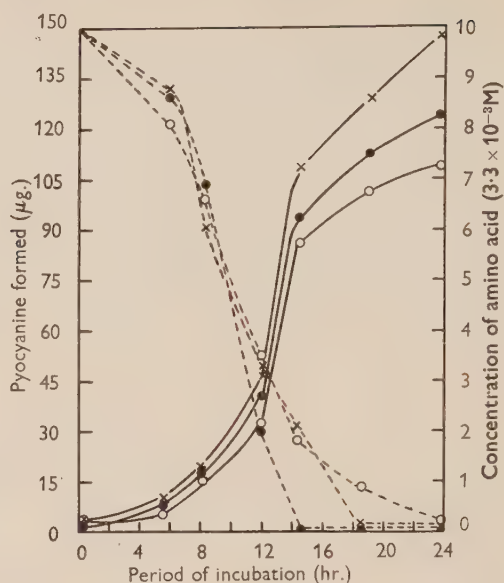


Fig. 6

Fig. 5. Influence of glutamic acid concentration on the formation of pyocyanine. Total volume: 1 ml.; organisms equivalent 4.7 mg. dry wt. bacteria; phosphate buffer, pH 7.8, 0.02M.

Fig. 6. The relationship between substrate disappearance and the formation of pyocyanine. Total volume: 1 ml.; amino acids, 0.03M; MgSO_4 , 0.02M. Phosphate buffer, pH 7.8, 0.02M. Incubation at 37°. \times = γ -aminobutyric acid; \circ = L-glutamic acid; \bullet = L-hydroxyproline. Solid line = pigment formation; broken line = disappearance of substrate.

DISCUSSION

Glutamic acid and some compounds closely related to it were thus found to be very good substrates for pyocyanine formation by non-proliferating suspensions of *Pseudomonas aeruginosa*. The greater production of pyocyanine in the presence of γ -aminobutyric acid than in the presence of glutamic acid raises the question as to whether the γ -aminobutyric acid serves as a suitable precursor in the biosynthesis or whether it enters the organisms more rapidly than glutamic acid and is there carboxylated to glutamic acid. The high yield of pyocyanine in the presence of β -alanine (the carboxylation of which to aspartate is not known to occur in this organism), suggests that the decarboxylated amino acids (β -alanine, γ -aminobutyric acid) may function as such and not via glutamic or aspartic acids. On the other hand, the appreciable activity of α -alanine might be explained by the ability of this compound to enter the Krebs cycle via pyruvic acid, and subsequently to undergo transformation to glutamic acid. The same mechanism might explain the effect of acetic, fumaric and succinic acids. This possibility appears plausible in view of the occurrence of the tricarboxylic acid cycle in various species of *Pseudomonas* (Barrett & Kallio, 1953; Campbell, Smith & Eagles, 1953; Claridge &

Werkman, 1954). The present work supports the view that glutamic acid holds a key position in pyocyanine synthesis and may be linked with the tricarboxylic acid cycle. Some insight into the sequence of the biosynthetic steps of pyocyanine formation may be gained by studying the relationship between the disappearance of the substrate and the formation of pyocyanine. Fig. 6 shows that the bulk of the substrates disappeared before measurable amounts of pyocyanine were formed and its synthesis continued after complete exhaustion of the added substrates. This relationship points towards the formation, from glutamic acid, of some unknown intermediary prior to its incorporation to form the molecule of pyocyanine.

The role of magnesium ions in pyocyanine formation was demonstrated in a magnesium-deficient system. The minimal effective amount of Mg^{++} ions was lower with glutamic acid than with any of the other substrates tested. The steep ascent of the curve resulting from the addition of Mg^{++} beyond 5–6 μ mol/ml. (Fig. 1) suggests that this ion participates in more than one reaction step.

The rate of pyocyanine synthesis was rather slow. Attempts to enhance its rate of formation by increasing aeration, or by supplying missing nutrients, have so far failed. It might perhaps be assumed that some essential intermediary of unknown nature, which is synthesized at a very slow rate, acts as a limiting factor in the formation of pyocyanine. The specific sensitivity of the system to respiratory poisons suggests a function of respiratory enzymes in pyocyanine biosynthesis.

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The Active Agent in Nascent Phage Lysis of Streptococci

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SUMMARY: A powerful lytic factor has been obtained in phage lysates of group C streptococci, which is active against streptococci of groups A, C and E and under some conditions group H. It is the factor responsible for 'nascent' phage lysis. The lytic activity remains unaltered by the removal of the phage by high-speed centrifuging, and also in the presence of phage antiserum. It is active against young and old cell suspensions, live, or killed by chloroform. The activity diminishes in the absence of reducing agents and it is destroyed by proteolytic enzymes. Heat-killed cocci when attacked by the lytic factor become Gram-negative but do not lyse. The addition of a proteolytic enzyme completes lysis. Efforts to demonstrate the release of proteinases from streptococcal suspensions have failed. After lysis the group polysaccharide is free as a hapten and some cell-wall structure remains. M antigen is also present in group A lysates.

In 1934 Evans reported that a phage, ordinarily lytic for group C but not for group A streptococci, lysed the group A cocci when sensitive group C streptococci were also present in the culture. No phage active on group A cocci could be recovered from the lysate. Evans considered that at the time of liberation the group C phage had a wider range of activity than later and referred to the phenomenon as 'nascent lysis'. This paper reports a re-investigation of the cause of nascent lysis, using the same phage as Evans. A powerful lytic agent was obtained from the lysates and this was studied to establish its origin and role in a 'nascent phage' system.

METHODS

The phage used was Evans's original phage B563, specifically active for group C streptococci. It proved to be very active on a stock group C strain Azgazardah (Griffith's original type 7) and this strain was used for propagation throughout.

Most of the group A strains employed were Griffith's original type strains kept lyophilized in this laboratory. Strains of other groups were from our stock collection originating from many sources.

The proteolytic enzymes used were: crystalline trypsin (Armour and Co. Ltd.); ficin and papain supplied in powder form (L. Light and Co. Ltd.); crystalline streptococcal proteinase generously supplied by Dr S. D. Elliott.

Phage propagation. A warmed 50 ml. volume of nutrient broth was seeded with 0.5 ml. of an 18 hr. broth culture of strain Azgazardah. After 2 hr. incubation, 1 ml. of stock phage suspension was added and incubation continued. The turbidity which had developed was cleared by phage action within $2\frac{1}{2}$ hr.

Nascent lysis. To 18 hr. broth cultures of the phage-resistant strain under investigation, 2–5% (v/v) of an 18 hr. culture of the propagating strain was added. The mixture was used as an inoculum for broth or flooded over solid media to give a bacterial lawn. Lysis of the resistant strain upon the addition of phage to the mixture was termed 'nascent lysis'.

Testing of the lytic agent. Streptococcal cultures were concentrated by centrifugation so that approximately 3 drops from a 0.02 ml. dropping pipette suspended in 1 ml. of broth or phage lysate gave an optical density equal to an 18 hr. glucose broth culture, i.e. about 25×10^7 viable units/ml. A reducing agent, 0.1 ml. of a 2% solution of neutralized thioglycollic (mercaptoacetic) acid, was added and the tubes placed in a water bath at 37°. The results, read by eye, were recorded as +, ++, +++, +++++, to denote the progressive decrease in opacity with complete clearing of the suspension as the end-point.

RESULTS

Most of Evans's work was performed with broth cultures, and she reported that phage B563, specific in its filtered state for group C organisms, was lytic in its nascent state for all group A strains and for some members of group E. In preliminary experiments in this laboratory the filtered phage could not be propagated on, or adapted to, any of the group A strains tested.

Nascent phage reactions on solid media

A large number of group A strains and some members of all the other Lancefield groups were tested on solid media for susceptibility to nascent lysis. Bacterial lawns were made with the test strain either alone or mixed with 5% (v/v) Azgazardah culture on nutrient agar containing 0.5% glucose and 5% (v/v) horse serum. The stock phage was spotted on undiluted and in tenfold dilutions up to 10^{-5} . All group A strains and both the group E strains tested were lysed when the group C cocci were present. Table 1 shows results representative of those obtained throughout.

The fate of resistant group A strains during phage propagation

The phage-propagating strain Azgazardah always gave a small compact colonial form on glucose serum agar, and could be readily distinguished from a strain of group A, type 6 (no. 8306) which formed large mucoid colonies on this medium. These two strains were incubated in broth separately and mixed, in both cases with and without phage. The six tubes were incubated and at intervals suitable dilutions were plated on serum glucose agar. After overnight incubation the group A and the group C colonies were counted (Fig. 1). Phage alone had no effect on the growth rate of the group A strain alone. In the mixture of groups A and C cocci, with phage present, both strains grew during the first hour and subsequently both were lysed but the numbers of group C organisms began to decrease first.

When the samples were quite clear they were tested for the presence of group polysaccharide by layering on groups A and C antisera. In the mixed

Table 1. *Nascent lysis on solid media of phage-resistant streptococci of different Lancefield groups*

Test strain	Bacterial lawn of test strain									
	Alone			+ 5 % of group C cocci						
	Phage dilution									
	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
Group A	-	-	-	-	-	+	+	+	-	-
C	+	+	+	+	+	+	+	+	+	+
E	-	-	-	-	-	+	+	+	+	+
B, D, F, G, H,	-	-	-	-	-	+	+	+	+	+
K, L, M, N, O	-	-	-	-	-	+	+	+	+	+

- = no plaques; + = 0.20 plaques; ++ = 50-100 plaques; +++ = 50 % lysis; ++++ = confluent lysis.

Table 2. *Action of lytic factor on representative strains of all Lancefield groups of streptococci used as bacterial lawns and in suspensions*

Streptococci of group	Dilution of lytic factor spotted on to bacterial lawn							Broth suspension added to reduced lytic factor
	0	10	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
	Degree of lysis							
A	+	+	+	+	+	+	+	Lysed
C	+	+	+	+	+	+	+	Lysed
E	+	+	+	+	+	+	+	Lysed
H	+	+	+	+	+	+	+	Lysed
B, D, F, G, H,	-	-	-	-	-	-	-	Lysed
K, L, M, N, O	-	-	-	-	-	-	-	Not lysed

Symbols as in Table 1; (++++) indicates secondary growth in the lysed area.

culture with phage, both group A and group C polysaccharides were present. The supernatant fluid of the control cultures, without phage, failed to react with antisera. There was therefore no doubt that the group A organisms were lysed and that the 'nascent phage' effect was not, as suggested by Whitehead, East & McIntosh (1953) for the *Streptococcus lactis* phage system, the result of inhibition of the growth of the group A strain by large numbers of phage particles adsorbed on to each coccus. At the same time the lysate of the mixed culture, when filtered, contained phage active only on the group C strain. A second experiment with a group A type 24 strain gave a similar result.

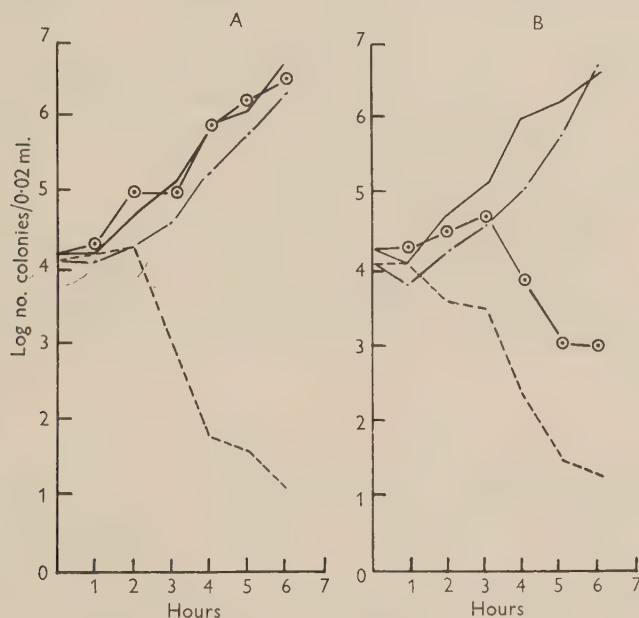


Fig. 1. Nascent lysis of group A streptococci in the presence of group C streptococci and group C phage, as shown by counts of viable units. Group A streptococci alone, —; group A streptococci + phage, ○—○; group C streptococci alone, — — —; group C streptococci + phage, - - - -. A. Both strains growing separately with or without phage. B. Both strains in the same culture with or without phage.

A lytic factor in filtered nascent lysates

The lysates produced during these experiments from the mixed culture and phage, and from the group C strain and phage, were later filtered and tested for lytic activity on group A cocci by adding a few drops of a well-grown culture of group A type 6 streptococci to 1 ml. of each filtrate and incubating in a water bath at 37°. Although the phage present was demonstrably not active on group A streptococci there was, nevertheless, some lysis of the suspension in both samples after 2–3 hr. The addition of 0.1 ml. of a 2% solution of sodium thioglycollate increased this lytic activity considerably.

When a freshly propagated group C phage lysate was filtered and tested in the same way, rapid lysis of a heavy suspension of group A cocci occurred.

This lytic power was a consistent feature of all the fresh group C phage filtrates, some of which could be diluted 1/500 and still show lytic activity against group A organisms. Undiluted samples lysed group A suspensions of 25×10^7 viable units/ml. in 2–3 min. Reduction by thioglycollate was only necessary with old samples or with fresh samples when the content of organic material had been diluted during the process of partial purification.

A lytic factor as the cause of nascent lysis

If the nascent lysis as observed by Evans depended on the formation of the lytic factor described in the previous section it should be possible to obtain lysis with phage filtrate alone on all groups of streptococci which had shown nascent lysis when mixed with the phage and its propagating strain. Heavy suspensions of cocci of all groups used earlier were mixed with active samples of lytic factor, reduced with thioglycollate, and put into a water bath at 37°. Members of groups A, C and E lysed readily, while group H cleared more slowly. None of the other strains was affected.

A potent lytic-factor filtrate produced confluent lysis of a group A bacterial lawn when applied undiluted or at a dilution of 1/10 (Table 2). There was no effect at all in higher dilutions which should have given discrete plaques if such strong lysis were due to phage action. The 'nascent-phage' effect could thus be obtained using a good lytic factor lysate in the absence of the propagating strain.

The role of the phage particle in nascent lysis

Once formed the lytic factor did not require the presence of phage for its activity. Because the phage was inactive on group A cocci and the coccal suspension comparatively old, it seemed unlikely that the nascent lysis could be due to phage multiplication. An antiserum prepared against group C phage, when added to phage filtrates, completely neutralized the phage activity but did not affect the activity of the lytic factor. Phage could also be removed by high-speed centrifugation and samples containing a high concentration of lytic factor with relatively few phage particles were prepared by this means. The centrifuged deposit was taken up in a small amount of broth, thus giving a sample containing little lytic factor but a large amount of phage. All these preparations were reduced and tested for lytic activity against various suspensions of group A type 6 cocci. These suspensions were (1) a live suspension in the log phase, and (2) an 18 hr. broth culture tested (a) untreated, (b) killed with chloroform, (c) killed by heating at 56°, (d) killed by heating at 85° (Table 3).

The lytic factor was active against live and chloroform-killed suspensions and against cocci killed by heating to 56° for 1 hr. Even when the phage content had been diminished to less than 1 particle/500 streptococci, lysis was still rapid. On the other hand, a heavy phage suspension containing little lytic factor was almost inactive. Cocci heated to 85° were almost entirely resistant and subsequently it was found that group A streptococci killed by steaming for 1 hr. were completely resistant to lysis. In ditch plates the lytic factor

diffused through the agar and remained lytic, but in a similar experiment the phage particles did not diffuse.

Table 3. *The influence of age and viability on the susceptibility of group A streptococci to lysis by the lytic factor with and without large numbers of phage particles present*

Condition of group A culture	Lysis produced by original lytic factor filtrate diluted		
	1/2	1/4	1/8
	Relative degree of lysis		
In log. phase, alive	++++/++++	++++/++++	+++ /++++
18 hr. culture, alive	++++/++++	++++/++++	++++/++++
Chloroform killed	++++/++++	++++/++++	++++/++++
Heated to 56°	+/+	+/+	-/+
Heated to 85°	±/±	±/±	-/±
Phage titre	$5 \times 10^9/\text{ml.}$		
Condition of group A culture	Lysis produced by supernatant after high-speed centrifugation diluted		
	1/2	1/4	1/8
	Relative degree of lysis		
In log. phase, alive	++++/++++	++++/++++	++++/++++
18 hr. culture, alive	++++/++++	++++/++++	++++/++++
Chloroform killed	++++/++++	++++/++++	++++/++++
Heated to 56°	+/++	+/++	±/++
Heated to 85°	±/±	±/±	±/±
Phage titre	$1 \times 10^4/\text{ml.}$		
Condition of group A culture	Lysis produced by phage deposit after high-speed centrifugation, resuspended in broth and diluted		
	1/2	1/4	1/8
	Relative degree of lysis		
In log. phase, alive	±/++	-/±	-/-
18 hr. culture, alive	±/++	-/±	-/-
Chloroform killed	+/++	+/++	-/±
Heated to 56°	-/±	-/-	-/-
Heated to 85°	-/±	-/-	-/-
Phage titre	$5 \times 10^{10}/\text{ml.}$		

The reading before the stroke was made after 15 min. at 37°, and the one after the stroke after 45 min. The symbols +, ++, +++, +++++ denote the progressive decrease in the opacity of the suspension.

Lysis of suspensions of heat-killed streptococci

Group A streptococci which had been killed by steaming were not lysed by lytic-factor filtrate, although chloroform-killed organisms were susceptible. Heat-killed organisms were, however, lysed by filtrate when trypsin was added. The trypsin could be replaced by 0.1 % of ficin, papain or streptococcal proteinase. Trypsin, ficin and papain incubated alone with the lytic factor destroyed its activity. The heated cocci were therefore usually pretreated with the lytic factor and the proteolytic agent added after incubation for 10 min.

The results so far obtained with the lytic factor on heat-killed cocci suggested that streptococcal proteinase or intracellular autolytic enzymes were destroyed by heat. It might be that the lytic factor attacked the cell wall but failed to lyse the cell and that replacement of the streptococcal enzymes by a proteolytic enzyme was required for dissolution of the cell. If this were the case a proteolytic agent ought to be detectable in the lytic-factor lysates of streptococci or in streptococcal suspensions under suitable conditions.

Repeated attempts were made to demonstrate such a proteinase in group A culture supernatant (fresh and after standing for 1 week in the presence of the cocci at room temperature), in group A cocci disintegrated in a Mickle disintegrator and in lytic factor lysates of group A organisms. Substrate was also added to a mixture of group A cocci + lytic factor during actual lysis. Freshly propagated phage filtrate was also tested for proteolysis. The substrates employed were casein, gelatin, streptococcal M antigen, and heat-killed group A cocci pretreated with lytic factor. The tests were carried out at pH values of 7.5, 7.0, 5.0 and 3.0. A test for the streptococcal proteinase described by Elliott was carried out with an antiproteinase serum which gave a good precipitate when mixed with the enzyme. Culture supernatants of strain 8302 did not contain active streptococcal proteinase. None of these preliminary tests showed the presence of any proteinase. That the cocci of heat-killed suspensions were attacked to some degree by lytic factor was shown by the release of the group polysaccharide into the supernatant; they also became Gram-negative. This appeared to be the first visible change in suspensions of any susceptible organism but was never seen in cells resistant to lytic factor.

Electron-microscope preparations of heat-killed suspensions of group A streptococci treated with lytic factor showed some evidence of damage but the cytoplasmic mass remained dense and in coccoid form owing to the heating (Pl. 1, fig. 1). On the other hand, when live organism + lytic factor mixtures were examined, cocci apparently devoid of cytoplasm were seen among otherwise complete chains (Pl. 1, fig. 2) and later only cell envelopes remained, some with a little cytoplasm still retained (Pl. 1, fig. 3).

Properties of the lytic factor

The lytic factor was active over a pH range of 6.5–8.6. Samples which were allowed to remain at a pH value below 4.0 or above 8.6 for some hours, and then readjusted to pH 7.5, were inactive. That the lytic factor was a protein was suggested by its destruction by proteinase. It was precipitated by 40 % saturation with ammonium sulphate; subsequent dialysis against tap water left a weak product which often increased in potency on the addition of ammonium sulphate. The crude lytic material could be freeze-dried without loss of activity.

It has not so far been possible to prepare an antiserum which neutralizes the lytic factor; all attempts have resulted in sera which neutralize the phage but completely fail to neutralize the lytic factor. Phage was always present in the material used as an antigen, and the inoculation of rabbits with the lytic-factor filtrate in which the phage was neutralized by antiphage serum also stimulated the production of antibodies to the phage.

The production of the lytic factor

The strength of the lytic factor produced during phage propagation seemed to depend on the amount of phage multiplication. Five propagations were made with a standard number of phage particles added to a varying number of organisms. A 50 ml. volume of warmed broth was seeded with a heavy inoculum of group C cocci and incubated for 4 hr. at 37°. A viable count was made and the culture then diluted 1/2, 1/5, 1/25 and 1/125 in broth. To 10 ml. volumes of each dilution and of the undiluted cultures 0.4 ml. of phage stock was added and the cultures incubated for 2 hr. All except the undiluted sample were quite clear at this time, and this sample cleared after being kept at 4° overnight. All samples (stored overnight at 4°) were then tested for phage titre and for the potency of the lytic factor. The phage concentration and the lytic activity were greatest in the 1/2 sample which had had 1.3×10^8 viable units/ml. and an initial bacterium:phage ratio of 13.7:1 (Table 4). The sample in which there had been the heaviest concentration of organisms and which had lysed rather slowly during the propagation period had a poor phage titre, and although very large numbers of cocci had lysed ultimately, the potency of the lytic factor was also very low.

In another experiment a constant number of cocci in the logarithmic growth phase were mixed with different concentrations of phage and the lytic factor activity and final phage content estimated. Although the initial bacterium:phage ratio ranged from 2.5:1 to 0.1:1, the lytic factor activity was approximately equal in all mixtures. The final concentration of phage particles in each sample was also equal (Table 5).

The conclusion reached from a number of experiments of a similar type was that the lytic factor was strongest when phage propagation was best and that this occurred where the number of propagating cocci was relatively large, with multiplicity of phage infection of 4. The lytic factor appeared soon after the beginning of lysis and was never present in streptococcal cultures or dis-integrates, unless phage lysis had taken place.

It seemed that at least a part of the lytic factor was produced by phage or by the streptococci under the influence of phage and was released at the time of release of phage. If it had been a product of lysed cocci alone it might have been possible to have built up the activity of a poor lytic-factor sample, from which phage had been removed, by the addition of streptococci at intervals as lysis was completed. The activity of the lytic factor was not increased by this procedure.

Dubos (1937) obtained from autolysing pneumococci an enzyme capable of causing pneumococci to become Gram-negative and Pirie (1939), with *Escherichia coli*, showed an enzyme present in culture supernatant fluids which attacked the coli bacterial polysaccharide. Efforts to find such an enzyme in cultures of the group C strain Azgazardah were unsuccessful.

In considering the source of the lytic factor it seemed worth while testing a number of group C strains for propagation of the phage to find whether the lytic factor was always produced when phage B563 multiplied, or whether it

Table 4. *Potency of lytic factor obtained after phage propagation using a constant number of phage particles with increasing numbers of viable streptococcal units*

Initial number of streptococci per phage particle	Final phage count (particles/ml. $\times 10^{-9}$)	Lytic factor diluted			
		Nil	Relative degree of lysis		
			1/10	1/20	1/40
27.5	0.035	±/+	-/+	-/tr	-/-
13.7	1.25	++	++	++	++
5.5	0.7	++	++	++	++
1	0.12	++	++	++	tr/++
0.2	0.06	++	-/+	-/+	-/+
		-/tr	-/-	-/-	-/-

The reading before the stroke was made after 15 min. and the one after the stroke was made after 45 min. at 37°. Symbols as in Table 3.

Table 5. *Potency of lytic factor obtained after phage propagation, using a constant number of streptococci and increasing size of phage inoculum*

Initial number of streptococci per phage particle	Final phage count (particles/ml. $\times 10^{-9}$)	Lytic factor diluted			
		Nil	Relative degree of lysis		
			1/10	1/20	1/40
2.5	1	++	++	++	++
0.5	1	++	++	++	++
0.16	0.9	++	++	++	++
0.1	1.3	++	++	++	++

Symbols as in Table 3.

was peculiar to its multiplication on the strain Azgazardah. From a large range of group C strains, 12 were selected as representing those whose characteristics of haemolysis, colony form, and host range, showed the greatest diversity. The phage was eventually adapted to all the strains, although it propagated much more successfully on some than on others. In each case active lytic factor was produced and the strength of the samples ran parallel with the number of phage particles liberated.

With the phage used, therefore, the lytic factor was produced from all susceptible streptococci. To test the effect of different phages on the one organism repeated attempts were made to obtain phages from some twenty group C strains and three samples of sewage, but none was isolated. However, another group C phage, originating from sewage and supplied by Dr Asheshov, also produced the lytic factor during propagation on a group C, strain 6, supplied with it. The stock phage B 563 used in all the other experiments also produced the lytic factor when propagated on strain 6. The two phages were both lytic phages and were both neutralized by antiserum with B 563. The two propagating strains, however, were different: Azgazardah was of human origin, while strain 6 was an animal strain.

DISCUSSION

The extremely active lytic agent described here is present in lysates of the group C streptococcus strain Azgazardah produced by the action of a lytic phage. This agent is capable of lysing live or chloroform-killed suspensions of streptococci of groups A, C and E, and it seems likely that the agent is responsible for the nascent lysis observed by Evans on streptococci belonging to these groups. Virulent phages active against group A streptococci and used in previous work (Maxted, 1955) also gave nascent lysis of otherwise resistant members of group A; such phage propagations also yielded a lytic principle similar to the one described here, although so far they have always been much less potent. This would agree with the observations of Evans that phage B 563 was by far the most active of her phages in respect of nascent lysis.

It is not yet clear whether the lytic factor is produced by the phage alone or whether it is a streptococcal product activated by the phage, or a streptococcal product that activates some substance liberated by the phage; it is, however, only demonstrable when phage lysis has taken place. A very large number of cocci could be lysed with a low multiplicity of phage infection, but unless the phage propagation was good the lytic factor potency was poor. It seems to be similar to the substance obtained by Wahl & Josse-Goichot (1950) from *Streptococcus lactis*. They suggested that their agent was a product of the streptococcus since the greater the proportions of organisms to phage in the propagation, the stronger the lytic agent. Weidel (1953) suggested that some phages may be a good source of cell-wall enzymes.

A dramatic characteristic of the lytic factor is the speed with which a potent sample will lyse comparatively heavy suspensions of live group A cocci. Lysis

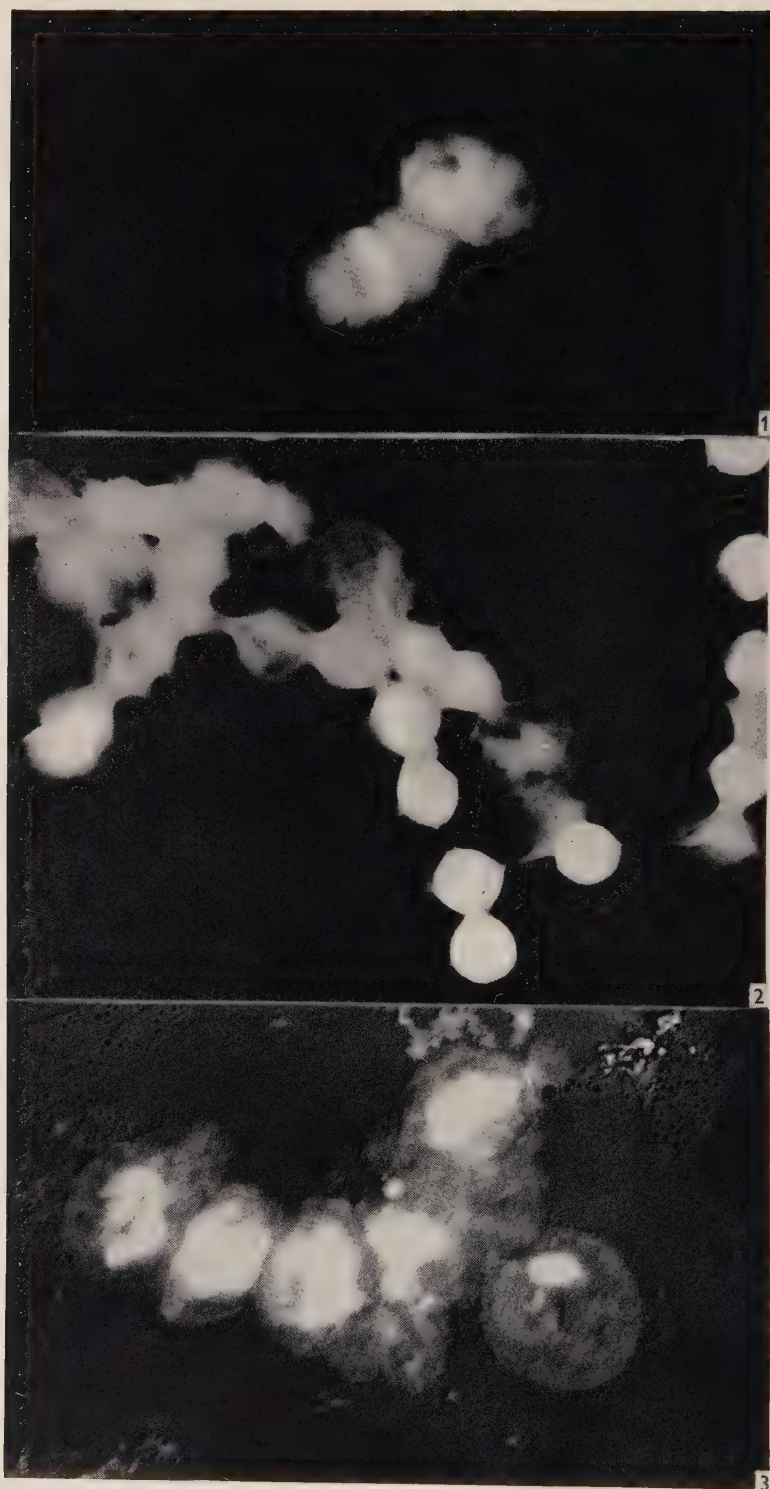
of phage-resistant organisms by this agent is certainly independent of the phage particle and is unlike some similar agents found by others in phage lysates of various other species.

A number of lysins have been described for Gram-negative bacilli, such as the lysin B of Sertic (1937) which destroys some surface antigens but does not lyse the bacilli, and the prolysins of Panijel & Huppert (1956) which lyse acetone treated bacteria but not living organisms growing in broth. Ralston, Baer, Lieberman & Krueger (1955) described a lytic principle, 'virolysin', found in phage lysates of some staphylococci, which could lyse staphylococci killed by heating to 56° but could only lyse live staphylococci in the logarithmic phase of growth when phage particles were present. On the other hand, the present streptococcal lytic factor readily lyses young live cultures but is much less active on heated suspensions and requires the addition of proteinase to complete the lysis of suspensions which have been heated above 80°. Pursuing an observation made by Dr Kjems of Copenhagen (private communication, 1955) it has been possible to demonstrate a lytic agent present in phage lysates of enterococci which is similar to 'virolysin', and differs from the lytic factor in the same way as 'virolysin' in its action on heat-killed organisms.

McCarty (1952) studied the lytic substances in the streptomyces filtrate that has been used to lyse haemolytic streptococci (Maxted, 1948) and suggested that there are two enzymes. One of these initiates the process by attacking the cell wall and thus prepares the cell for complete dissolution by the second, a proteolytic enzyme. There is an apparent analogy between the streptomyces lytic factor and the one described here, which shows a similar need for an added proteolytic factor in order to lyse heat-killed cocci. But it may be that with living streptococci no proteolytic activity is involved; certainly at present there is no evidence for major protein destruction. The great increase in the protein content in lytic factor lysates of streptococci, as shown by precipitation with trichloroacetic acid, suggests that after the initial attack on the cell wall or cell membrane the cytoplasmic material diffuses into the surrounding fluid. The cell walls of heated streptococci are also attacked, but the cytoplasmic material appears to remain intact perhaps because of the alteration of its physical state by heat. Preliminary electron microscope examination supports this view.

A single lytic agent of biological origin capable of lysing fast-growing streptococci, with so little apparent destruction of cell contents, should prove of some interest. It may prove a useful tool in the study of streptococci and it points at once to a difference between the structure or composition of groups A, C and E strains and those of other groups; it also offers a quick and simple means of obtaining intracellular elements. The use of purified lytic factor and purified cell-wall material prepared by mechanical means may disclose the site of attack and the part played in lysis by proteolytic agents. Work on this aspect is in hand.

The phage B563 used throughout this work is a virulent phage. There are possibly three kinds of phage active on group A streptococci: the virulent phages originating from sewage, temperate ones reported by Kjems (1955), and



W. R. MAXTED—NASCENT PHAGE LYSIS. PLATE 1

(Facing p. 595)

other temperate phages isolated here (Maxted, in preparation). There is, however, reason to believe that not all produce a lytic factor similar to the one described here and it may be that this phenomenon is a feature of the virulent streptococcal phages only.

My thanks are due to Dr R. C. Valentine of the National Institute for Medical Research for the electron micrographs, and for guidance in their interpretation.

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EXPLANATION OF PLATE

Electron micrographs; all preparations were fixed in osmium tetroxide; platinum shadowed.

Fig. 1. Appearance of heat-killed streptococci after treatment with lytic factor. $\times 25,000$.

Fig. 2. First stage in lysis of live streptococci by dilute lytic factor. $\times 15,000$.

Fig. 3. Residual structure remaining after fast lysis of living streptococci has taken place. $\times 30,000$.

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On the Osmotic Behaviour of *Desulphovibrio desulphuricans*

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SUMMARY: When known volumes of wet *Desulphovibrio desulphuricans* were suspended in standard NaCl solutions, the chloride was not diluted by intracellular water below 3 % (w/v) NaCl; at 4 and 6 % NaCl exchange occurred but not to the extent observed when the organisms were made permeable with cetyltrimethylammonium chloride (CTAC). With intact organisms Na_2SO_4 up to 15 % (w/v) was not diluted by intracellular water. Dilution of 4 and 6 % NaCl was due to plasmolytic dehydration of organisms rather than to osmotic breakdown, since phosphate, cytochrome c_3 and 265 m μ -absorbing material did not escape from the organisms, though they did with CTAC; plasmolysis was not visible microscopically. Hence these sulphate-reducing bacteria are freely permeable neither to chloride nor sulphate. Data on bacterial dimensions and fluid contents derived from this work are recorded; they compare satisfactorily with those obtained by optical measurements.

The question whether salts penetrate sulphate-reducing bacteria freely is of particular interest because their metabolism depends on the reduction of an anion. Roberts, Abelson, Cowie, Bolton & Britten (1955) recently claimed that *Escherichia coli* was freely permeable to anions such as phosphate and sulphate, though this finding was disputed by Mitchell & Moyle (1956). The present paper records a study of the osmotic behaviour of a strain of *Desulphovibrio desulphuricans* towards chloride and sulphate.

METHODS

Organism. A freshwater strain of *Desulphovibrio desulphuricans*, strain Wandle (National Collection of Industrial Bacteria, NCIB 8305) was used in this work. The strain was subcultured (0.05 ml. into 5 ml. medium) weekly in a lactate + yeast extract + sulphate medium (medium C of Butlin, Adams & Thomas, 1949) supplemented with cysteine (5 μ mole/ml.) to poise the oxidation-reduction potential (Grossman & Postgate, 1953*a*); viable counts were done in a similar medium set with agar (Grossman & Postgate, 1953*b*). Organisms for work with heavy suspensions were grown in 500 ml. or 2 l. flasks containing a similar medium supplemented with Tryptone (Oxoid; 0.5 %, w/v) and extra yeast extract to 0.4 % (w/v). The Tryptone was added to ensure maximum hydrogenase activity in connexion with work reported elsewhere (Littlewood & Postgate, 1956).

Standardization of bacterial suspensions. The concentration of bacterial suspensions was determined turbidimetrically in the E.E.L. colorimeter (Evans Electroselenium Ltd., Harlow, Essex) calibrated with the Hildenborough strain of *Desulphovibrio desulphuricans*.

Spectrophotometric measurements. These were made in the 'Uvispek' instrument (Hilger and Watts, London) with 1 cm. optical cells.

Osmotic behaviour of bacteria. The 'thick suspension' technique of Mitchell & Moyle (1956) was used to determine whether external chloride or sulphate came to osmotic equilibrium with the intracellular water. For the experiments with chloride, washed bacterial suspensions of known concentration in KH_2PO_4 (0.5 %, w/v, pH 6.3), were centrifuged in graduated conical centrifuge tubes to constant volume (2 hr.). The supernatant fluid was discarded and each pad of packed organisms resuspended in a volume of standard NaCl solution equal to that of the wet mass of organisms. After re-centrifuging, the extent of dilution of the chloride in the supernatant fluid was determined analytically (see below). For the experiments with sulphate the procedure differed from this in two respects: (1) since phosphate interferes with sulphate estimation, the bacteria were suspended in sodium acetate buffer (0.8 %, w/v, pH 6.5); (2) to minimize errors due to sulphate reduction during manipulation, the suspensions were centrifuged in plastic cups at a high speed (18,000 *g*) for *c.* 10 min. The volume of organisms in each cup was calculated from the relation
$$\frac{\text{volume of wet bacteria}}{\text{dry weight of bacteria}} = 5 \text{ ml./g.}$$
 obtained in the experiments with chloride. The organisms were then resuspended in 1 ml. volumes of standard Na_2SO_4 solutions; controls with NaCl were run simultaneously.

The total space penetrable by ions when close-packed bacteria were made completely permeable was determined by using salt solutions containing the cetyltrimethylammonium ion, which renders the organisms permeable to small molecules (Postgate, 1956) added as the chloride (CTAC; 100 $\mu\text{g.}$ /mg. dry wt. bacteria).

Estimation of anions. Chloride was estimated by titration against AgNO_3 , with potassium chromate as indicator (Stockdale & Dexter, 1938); in our hands this procedure gave a constant positive mean error of 2.49 % with a standard error of 0.31 %. Sulphate was estimated by the method of Schroeder (1933); this procedure also gave a small mean positive error, 1.86 %, with a standard error of 0.32 %. Phosphate was estimated by Fiske & SubbaRow's method as described by Umbreit, Burris & Stauffer (1949).

Manometry. The maximum rate of sulphate reduction at room temperature was estimated manometrically using the procedure described by Postgate (1951), except that the bacterial suspensions were pre-incubated in hydrogen for 2-3 hr. before adding the substrate.

Reagents. Analar reagents were used where available. CTAC was prepared by passing a solution of cetyltrimethylammonium bromide through the basic form of Amberlite IR-400 (British Drug Houses Ltd.). The effluent was neutralized with HCl, frozen and evaporated to dryness; the product was refluxed with acetone, and the NaCl removed by filtration. After cooling, the acetone was filtered off, and the remaining traces of acetone removed by evaporation in a vacuum oven.

Optical measurements. As a check on values derived from solute penetration measurements, certain dimensions of the living bacteria were measured.

Knaysi (1944) discussed the considerable errors inherent in optical measurements on fixed and stained cells, and similarly drying and shadowing procedures can introduce errors in measurements on electron micrographs. The dimensions recorded in this paper were therefore obtained optically under phase contrast with a micrometer eyepiece. The bacteria were grown in a liquid medium, centrifuged, set in warm agar (1.5%) in 0.8% NaCl (to inhibit Brownian movement and motility) and slides were prepared from droplets of this agar suspension. Quoted readings are the means of ten organisms measured.

For calculating cell volumes, etc., the organisms were regarded as flat-ended cylinders. With this particular strain neglect of the vibrio form introduced little error, since the organisms were short, their curvature slight, and no spirilloid forms were present. This assumption would have introduced a much greater error with a more curved or spirilloid strain.

Units. Concentrations of salt solutions are expressed as % (w/v); ion-penetrable volumes are expressed as % (v/v) of the total volume of wet close-packed bacteria; units of cytochrome c_3 are those used by Postgate (1956).

RESULTS

Osmotic behaviour towards chloride and sulphate. The osmotic behaviour of *Desulphovibrio desulphuricans* strain Wandle towards chloride, investigated by the heavy suspension technique, is indicated in Table 1. In 0.5–3% NaCl, the space penetrated by chloride (V_{cr}) was approximately constant; it greatly increased in 4% or more NaCl. This suggests that 0.5–3% solutions were only diluted by intercellular water, but in 4% or stronger NaCl solutions the chloride ion was diluted by intracellular water as well. The value of V_{cr} obtained with a NaCl+CTAC mixture equivalent in chloride to 1% NaCl was higher than the highest values obtained for V_{cr} with 4 or 6% NaCl.

To investigate the osmotic behaviour towards sulphate, the technique was modified (see Methods). The maximum rate of sulphate reduction during centrifugation as determined by manometric controls was negligible; the figures given in Table 1 have been corrected for it. The extent of dilution of 1.5–15% Na_2SO_4 solutions was approximately constant and equivalent to that of a 2% NaCl solution, indicating that these Na_2SO_4 solutions were only diluted by intercellular water. With this modified technique higher values were obtained for the volume of intercellular water than were obtained with the earlier procedure. This is probably because the bacteria had not been centrifuged to constant volume; higher ion-penetrable volumes were also obtained using this technique when the bacteria were made permeable with CTAC, and when a control experiment was run with NaCl concentrations comparable to those used in the earlier experiments. This experiment gave the following results: 2% NaCl, $V_{cr}=40.4\%$; 4% NaCl, $V_{cr}=52.8\%$; 6% NaCl, $V_{cr}=58.8\%$; NaCl+CTAC, $V_{cr}=73.4\%$. The relationships of the values of V_{cr} agree substantially with those obtained earlier, indicating that the short duration of the experiments nevertheless allowed the organisms to reach osmotic equilibrium with chloride.

Table 1. *Apparent intercellular space in wet packed cells of Desulphovibrio desulphuricans strain Wandle*

Centrifuged bacteria of measured or calculated total volume (c. 1 ml.) were suspended in known volumes of standard salt solutions, the suspensions re-centrifuged and the supernatant fluids analysed for dilution of anion. The figures quoted are the space penetrated by the anion as % (v/v) of the total wet volume. The values for Na_2SO_4 are corrected for the maximum rate of sulphate reduction during the centrifuging procedure as determined by manometric controls.

Suspending fluid								NaCl+CTAC (see text)
Expt.	NaCl (% w/v)							
	0.5	1	2	3	4	6		
1	27.6	30.9	—	27.78	46.95	—	—	
2	27.28	27.93	32	—	46.06	—	—	
3	—	—	—	27.9	42.64	—	—	
4	—	—	—	33.61	49.95	—	—	
5	—	—	33.65	—	49.7	54.76	64.06	
6	—	—	—	—	52.28	—	65.81	

Suspending fluid									Na ₂ SO ₄ (3 %)+CTAC (see text)
Expt.	NaCl (% w/v)	Na ₂ SO ₄ (% w/v)							
	2	1.5	3	4	5	7	10	15	
7	35.9	38.2	34.76	39.44	—	—	—	—	—
8	37.52	38	40.13	40.07	—	—	—	—	—
9	38.3	—	39.07	—	39.28	38.47	—	—	—
10	37.72	—	37.07	—	—	—	—	—	77.9
11	38.23	—	38.8	—	—	—	38.41	—	73.83
12	36.08	—	34.36	—	—	—	38.1	—	74.61
13	38.53	—	37.45	—	—	—	—	38.2	—

Attempts to detect leakage of cellular constituents

Since 4 and 6% NaCl solutions exchanged with the intracellular water of strain Wandle it was possible that breakdown of the bacterial osmotic barrier had occurred, allowing soluble constituents of the cytoplasm to leak into the medium. Osmotic breakdown of this kind is characteristic of the action of quaternary detergents (Salton, 1951; Newton, 1956). The question was investigated by seeking (a) material which absorbed at $265\text{ m}\mu$; (b) cytochrome c_3 (Postgate, 1956); (c) inorganic phosphate in the supernatant fluid after organisms had been treated with NaCl at various concentrations. Controls were run in which bacteria were treated with CTAC, known to release soluble molecules having molecular weights up to that of cytochrome c_3 .

Materials absorbing at $265\text{ m}\mu$. No evidence was obtained for release of material absorbing at this wavelength (Fig. 1) on treatment with NaCl, but the experiments were not wholly conclusive because these bacteria, even after being washed four times, secreted material which absorbed at $275\text{ m}\mu$, which would mask small amounts of $265\text{ m}\mu$ material. The amount of substance absorbing at $275\text{ m}\mu$ was not related to the NaCl concentration, suggesting that it was not a product of osmotic leakage. The peak of absorption at $275\text{ m}\mu$ in fluid derived from CTAC-treated bacteria had the 'shoulder' at about

265 m μ . which would be expected were material absorbing at this latter wavelength released.

Cytochrome c_3 . Cytochrome c_3 was sought by its strong Soret band at 419 m μ . when in the reduced form. It was not detected in the supernatant

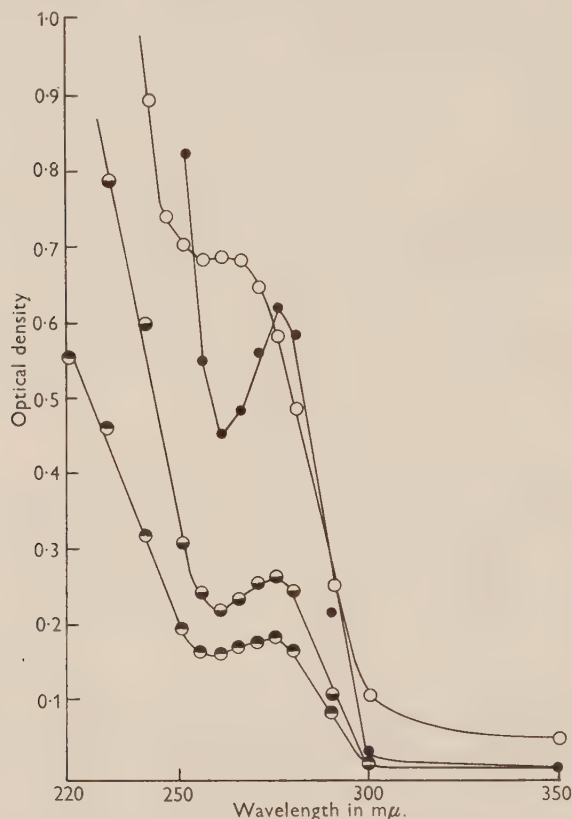


Fig. 1. Absorption spectra of supernatant fluids from *Desulphovibrio desulphuricans* strain Wandle treated with sodium chloride solutions. Samples of a suspension containing 5 mg. dry wt. organisms/ml. were exposed to NaCl solutions, centrifuged, and the supernatant fluids examined for material which absorbed at 265 m μ . A control with cetyltrimethylammonium chloride (CTAC) was included to release all soluble cellular constituents. \circ , 0.8 % NaCl; \bullet , 4 % NaCl; \ominus , 6 % NaCl; \circ , 0.8 % NaCl + 100 μ g. CTAC/mg. dry wt. organisms; total chloride \equiv 0.81 % NaCl.

fluid from bacteria (5 mg. dry wt./ml.) treated with 0.8, 4 and 6 % NaCl; it was present in the fluid from bacteria treated with CTAC, to between 0.42 and 0.53 m μ U. c_3 /mg. dry wt. organisms.

Phosphate. Small amounts of phosphate (1.1–2.6 m μ mole PO_4 /mg. dry wt. bacteria) appeared in the supernatant fluid when bacteria, washed four times in tris-(hydroxymethyl)-aminomethane buffer (pH 7.2) were treated with 0.8, 4 and 6 % NaCl; the amount of phosphate released was not related to NaCl concentration and was insignificant compared with the amount that appeared when organisms were treated with CTAC (17.2 m μ mole PO_4 /mg. dry wt. bacteria).

Attempts to observe plasmolysis. The tests described in the last section indicate that the dilution by intracellular water that took place when strain Wandle was exposed to 4% or 6% NaCl did not occur by breakdown of the osmotic barrier such as that induced by CTAC; no release of intracellular constituents occurred. Hence the process must be plasmolytic, but no clear retraction of the cytoplasm could be observed under phase contrast even with 25% NaCl. Plasmolysis was readily observed in control experiments with some unidentified Gram-negative freshwater pseudomonads and spirilla.

Effect of NaCl on viability. Exposure to 6% NaCl did not significantly alter the numbers of viable organisms present. A count on a suspension containing 1.2×10^9 viable organisms/ml. 0.8% NaCl gave a value of 1.4×10^9 viable organisms/ml. after exposure to 6% NaCl; CTAC reduced the viable population of a similar suspension to 7.1×10^6 viable organisms/ml.

Bacterial volume. For comparison with the dimensions obtained from the heavy suspension experiments (see Discussion) the mean dimensions of organisms in a 3-day culture of strain Wandle were measured microscopically. The mean length was 2.34μ . ($\sigma = 0.51 \mu$.) and the mean diameter was 0.74μ . ($\sigma = 0.043 \mu$.); hence the mean volume of an organism was $1.01 \mu^3$.

DISCUSSION

(i) *Penetration of anions.* True permeability is reversible in the sense that soluble molecules can diffuse freely in and out of the organism. Except when the organisms were deliberately rendered permeable with CTAC, penetration of this kind was not detected in the experiments reported here: neither sodium sulphate nor chloride at concentrations lower than 3% became diluted with intracellular water. Since the sulphate ion is the normal respiratory substrate of these bacteria it is therefore likely either that sulphate reduction occurs outside the osmotic barrier, or that penetration of sulphate into the organism is regulated in such a manner that the ratio of the equilibrium internal concentration of sulphate ions to the external concentration is so low that the analytical procedure used in this work could not detect penetration of the ion even at high external sulphate concentrations.

Sodium chloride at 4 and 6% induced considerable dehydration of the cells, without true penetration or loss of viability, since the chloride became diluted with intracellular water. Hence a process akin to plasmolysis must have occurred though we were unable to observe it microscopically. Such dehydration did not occur even with very high concentrations of Na_2SO_4 . The experiments reported here provide no explanation of this observation, which may be connected with metabolic importance of sulphate for these organisms. The impermeability of these bacteria to anions is in accord with studies by Mitchell & Moyle (1956) on other bacteria, and contrasts with the reports of Roberts *et al.* (1955) that *Escherichia coli* is freely permeable to ions such as sulphate.

(ii) *Dimensions of Desulphovibrio desulphuricans strain Wandle.* The data obtained in the heavy suspension experiments can be used to calculate certain dimensions of these organisms which, when compared with optical measure-

ments, provide a check on the validity of the technique used. The intercellular space among vibrios centrifuged to constant volume ranged from 27.3 to 33.6 % of the total volume (mean = 29.5 %, compare 25.5–28.3 % obtained by Mitchell (1953) from experiments on phosphate penetration with *Micrococcus pyogenes* (*Staphylococcus aureus*), and the theoretical value of 26 % calculated by Conway & Downey (1950) for close-packed spheres). One ml. of close-packed vibrios contained 6.5×10^{11} (total), hence the mean volume of a vibrio was $1.086 \mu^3$. Microscopic measurements gave a value of $1.01 \mu^3$ (Table 2). These values agree remarkably well, particularly since the microscopic measurements made no allowance for the slight curvature of the organisms, and since the heavy suspension experiments made no allowance for any solute-permeable part of the organism which might be outside the osmotic barrier. The agreement of the values confirms the opinion that the osmotic barrier to the ions studied here lies in the neighbourhood of the cell wall.

Table 2. *Comparison of mean morphological dimensions of Desulphovibrio desulphuricans strain Wandle obtained by optical and heavy suspension techniques*

For details of procedure see text. The organisms were regarded as straight flat-ended cylinders for volume and area calculations.

Dimension	From optical measurements	From heavy suspension experiments
Length	2.34 μ .	2.41 μ .
Diameter	0.74 μ .	0.76 μ .
Volume	1.01 μ^3 .	1.086 μ^3 .
Area	5.49 μ^2 .	5.81 μ^2 .
Dry weight	0.3125×10^{-12} g.	
Percentage intracellular protein	31.5 % (w/v)* 31 % (w/v)†	35.5 % (w/v)

* From refractive index. † From ratio of dry weight to volume.

The length and diameter of the vibrios obtained from optical measurements will be subject to similar errors, but their ratio will not; when this ratio is used to calculate the mean lengths and diameter from the volume obtained in the heavy suspension experiments, the mean area of an organism can be calculated. Values for each of these quantities are quoted in Table 2.

The dry weight of a single vibrio quoted in Table 2 was obtained by dividing the dry weight of a suspension by the total count. All these figures are in the range of those recorded by Knaysi (1944) for other bacteria, and are consistent with optical measurements made by Senez (to be published) on *Desulphovibrio desulphuricans* strain Canet 41. Table 2 includes figures from which a further comparison can be made: organisms treated with CTAC had a mean solute-impermeable residue of 35 % of their wet volume; i.e. 50.5 % of their own volume. If this residue be assumed to be protein of specific volume 0.71, then the concentration of protein in the undamaged organism should not exceed 35.5 % (w/v). Measurements of the cytoplasmic refractive index of strain Wandle in the manner used by Postgate (1956) for *D. desulphuricans* strain

Hildenborough gave a mean value of 1.383, corresponding to a 31.5% (w/v) solution of plasma albumin. These values are in fair agreement considering the assumption involved, and are consistent with the value of *c.* 31% obtained by dividing the mean optical volume by the mean dry weight. The value of the dry weight : wet volume ratio of 5 indicates that the proportion of solid matter in the wet packed organisms was *c.* 20% (w/v); Roberts *et al.* obtained a value of 22.3% (w/v) from dry-weight determinations on *Escherichia coli*.

We are indebted to Dr P. Mitchell for valuable discussions, to our colleagues of the Microbiology Group for advice and criticism during this work, and to Dr J. C. Senéz for supplying and discussing his data on the cellular dimensions of *Desulphovibrio desulphuricans* strain Canet 41. This paper is published by permission of the Director, Chemical Research Laboratory.

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The Synergistic Action of Cephalosporin C and Benzylpenicillin against a Penicillinase-producing Strain of *Staphylococcus aureus*

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SUMMARY: Cephalosporin C, a hydrophilic, penicillin-like antibiotic which is insensitive to penicillinase, is much less active than benzylpenicillin against the Oxford strain of *Staphylococcus aureus* but much more active than benzylpenicillin against a penicillinase-producing strain of *S. aureus* (D3R). When used together, cephalosporin C and benzylpenicillin act synergistically against *S. aureus* strain D3R *in vitro*, presumably because cephalosporin C is a competitive inhibitor of penicillinase. When grown under certain conditions in the presence of cephalosporin C, benzylpenicillin, or bacitracin, the Oxford staphylococcus gives rise during a single culture to a resistant bacterial population consisting largely or partly of Gram-negative bacilli. In the presence of bacitracin a similar change also occurs with *S. aureus* strain D3R.

Two hydrophilic antibiotics, cephalosporin N and cephalosporin C, have been isolated from the culture fluid of a species of *Cephalosporium* (Abraham, Newton & Hale, 1954; Newton & Abraham, 1956). Cephalosporin N is a new type of penicillin with a side-chain derived from D- α -aminoadipic acid (Newton & Abraham, 1954), and appears to be identical with synnematin B (Abraham *et al.* 1955). Cephalosporin C is chemically related to cephalosporin N, but is not a true penicillin (Abraham & Newton, 1956*a*). It is not sensitive to penicillinase, but is a competitive inhibitor of the action of this enzyme on penicillin (Abraham & Newton, 1956*b*), and it induces the formation of penicillinase by *Bacillus cereus* (Pollock, 1957). It has a low antibacterial activity, but no significant toxicity to mice, and it affords protection to mice infected with *Streptococcus pyogenes* or with a penicillin-resistant strain of *Staphylococcus aureus* (Florey, 1955, 1957).

The finding that cephalosporin C was a competitive inhibitor of penicillinase raised the question whether it would act synergistically with penicillin against penicillin-resistant staphylococci which produce this enzyme. The work described in the present paper was begun with the object of obtaining an answer to this question. During the work it was observed that staphylococci underwent profound morphological and biochemical changes when grown under certain conditions in media containing cephalosporin C, benzylpenicillin, or bacitracin.

METHODS

Materials. Benzylpenicillin (1650 units/mg., Glaxo Laboratories Ltd.) and cephalosporin C (Newton & Abraham, 1956) were used as the crystalline sodium salts. The bacitracin was a commercial sample (A/S Apotekernes

Laboratorium, Oslo) with an activity of 65 units/mg.; it consisted mainly of bacitracin A together with some bacitracin B. Crystalline micrococcin (Heatley & Doery, 1951; Abraham, Heatley, Brookes, Fuller & Walker, 1956) was kindly provided by Dr N. G. Heatley.

A penicillin-sensitive strain of *Staphylococcus aureus* (Oxford strain, NCTC 6571) and a resistant strain which produced penicillinase (strain D3R, phage type 6/47+; kindly provided by Dr Mary Barber) were used as test organisms. The penicillin-resistant strain D3R appeared not to produce a significant amount of an enzyme able to destroy cephalosporin C (cf. Abraham & Newton, 1956*b*). The culture medium used was in most cases a tryptic heart digest broth (TMB; Barnes, 1955). One batch of medium was used in many experiments.

Measurements of antibacterial activity

(1) Two-fold serial dilution tests were carried out at 37° in the conventional manner, the lowest concentration of antibiotic which prevented visible growth for different periods of time being recorded. In tests with mixtures of antibiotics the concentration of each component of the mixture was varied (in two-fold steps) independently.

(2) Aerated cultures from large inocula (which gave about 10^7 cocci/ml.) were grown in inverted T-tubes of the type devised by Dr J. Monod (see Kay & Fildes, 1950). The tubes were immersed in a water bath at 37° and attached to an apparatus by which they were rocked 44 times/min. Changes in the opacity were measured in a 'Spekker' absorptiometer (Adam Hilger Ltd.), a neutral grey filter (H508) being used. The inoculum was taken from an overnight stagnant culture in TMB. The antibiotic was usually added about 1.5 hr. after inoculation, when growth had just entered the logarithmic phase.

RESULTS

Activity of cephalosporin C and benzylpenicillin against different strains of *Staphylococcus aureus*

Serial dilution tests. Table 1 shows the effect of cephalosporin C alone, benzylpenicillin alone, and mixtures of the two on the growth of the penicillin-sensitive (Oxford) strain of *Staphylococcus aureus*. It is evident that benzylpenicillin was more than 1000 times as active as cephalosporin C against this

Table 1. *Inhibition of growth of Staphylococcus aureus (Oxford strain)*

The inoculum was 1 drop/ml. of an overnight TMB culture diluted 1/1000. The bracketed figures show the concentrations for various inhibitory mixtures of benzylpenicillin (upper values) and cephalosporin C (lower values). In general, growth occurred when the concentration of either component of an inhibitory mixture was halved.

Antibiotic	Concentration (μ g./ml.) inhibitory to growth for			
	1 day	2 days	3 days	6 days
Benzylpenicillin alone	0.015	0.015	0.03	0.06
Cephalosporin C alone	50	100	100	100
Benzylpenicillin	0.015	0.015	0.015	0.015
+ Cephalosporin C	12.5	50	50	50

organism under the conditions used; that the concentration of either compound required to inhibit growth for 6 days was not very much greater than that required to inhibit growth for 1 day; and that the activity of a mixture of the two compounds was not significantly greater than would be predicted on the assumption that their effect together was purely additive.

Tables 2 and 3 show the results of two similar experiments in which the penicillinase-producing *Staphylococcus aureus* (D3R) was the test organism. In one case (Table 3) the inoculum was 100 times as large as in the other. The larger inoculum gave about 10^6 cocci/ml. Benzylpenicillin was much more active against the smaller inoculum than against the larger one, and with both inocula it was much more effective in suppressing growth for 1 day than for 5 or 6 days. In contrast, the activity of cephalosporin C varied little with the size of the inoculum or with the time at which the end-point was recorded. With the small inoculum cephalosporin C appeared to be 5 times as active as benzylpenicillin after 6 days and with the large inoculum it appeared to be 20 times as active after 5 days. In both cases the activities of mixtures of cephalosporin C and benzylpenicillin were considerably greater than the sum of the activities of their components.

Table 2. *Inhibition of growth of Staphylococcus aureus strain D3R (penicillinase producer)*

The inoculum was 1 drop/ml. of an overnight TMB culture diluted 1/1000. The bracketed figures show the concentrations for various inhibitory mixtures of benzylpenicillin (upper values) and cephalosporin C (lower values). Readings after 6 days showed that growth occurred when the concentration of either component of an inhibitory mixture was halved.

Antibiotic	Concentration ($\mu\text{g./ml.}$) inhibitory to growth for			
	1 day	2 days	3 days	6 days
Benzylpenicillin alone	0.5	4	16	500
Cephalosporin C	100	100	100	100
Benzylpenicillin } + Cephalosporin C }	—	2 } 12.5 } 6.25 }	—	4 } 12.5 } 16 } 6.2 } 32 } 3.1 } 64 } 1.5 }

The organisms which grew up, in these tests, in the presence of the antibiotics appeared to be morphologically indistinguishable from normal staphylococci, except that they tended to be somewhat larger.

Changes in opacity of aerated cultures from large inocula. The results of a typical experiment in which cephalosporin C alone, benzylpenicillin alone and mixtures of the two antibiotics were added to cultures of the penicillinase-producing strain D3R of *Staphylococcus aureus* growing in aerated TMB are shown in Fig. 1. Under these conditions, the addition of the antibiotic caused no appreciable change in the rate of bacterial growth for about 1 hr. but, at the

end of that time, growth rapidly gave way to extensive lysis, leaving a culture which showed only a faint turbidity. After a period whose length depended on the initial concentration of the antibiotic, visible growth again took place.

Table 3. *Inhibition of growth of Staphylococcus aureus strain D3R (penicillinase producer)*

The inoculum was 1 drop/ml. of an overnight TMB culture diluted 1/10. The bracketed figures show the concentrations for various inhibitory mixtures of benzylpenicillin (upper values) and cephalosporin C (lower values). Readings after 3 or 5 days showed that growth occurred when the concentration of either component of an inhibitory mixture was halved.

Antibiotic	Concentration ($\mu\text{g./ml.}$) inhibitory to growth for			
	1 day	2 days	3 days	5 days
Benzylpenicillin alone	4	250	2000	4000
Cephalosporin C alone	100	200	200	200
Benzylpenicillin } + Cephalosporin C }	2 } 50 }	60 } 6.2 }	120 } 12.5 }	30 } 50 } 120 } 25 }

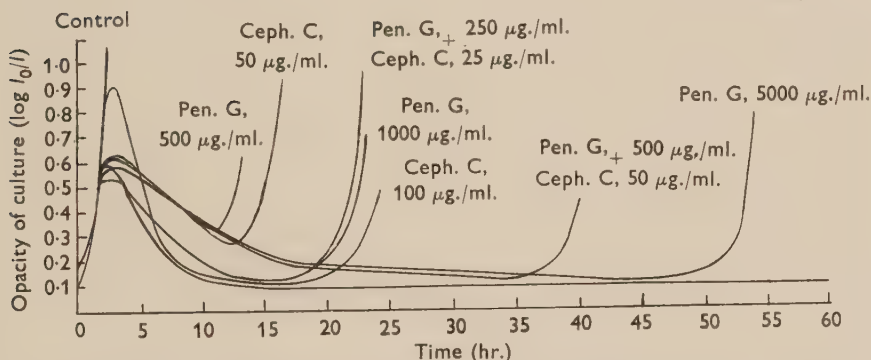


Fig. 1. Changes in the opacity of an aerated culture of *Staphylococcus aureus* strain D3R, on the addition of cephalosporin C alone, benzylpenicillin alone and mixtures of both antibiotics. \leftarrow shows the times at which the antibiotics were added. Pen. G = benzylpenicillin; ceph. C = cephalosporin C.

In terms of its ability to delay final growth, cephalosporin C was more than 10 times as active as benzylpenicillin against *Staphylococcus aureus* strain D3R. (In a similar experiment in which the medium was Difco heart infusion broth cephalosporin C was 40 times as active as benzylpenicillin against strain D3R.) When the two antibiotics were used together they acted synergistically. Thus, Fig. 1 shows that a mixture of 250 $\mu\text{g.}$ benzylpenicillin/ml. and 25 $\mu\text{g.}$ cephalosporin C/ml. was considerably more effective than 500 $\mu\text{g.}$ benzylpenicillin/ml. alone or 50 $\mu\text{g.}$ cephalosporin C/ml. alone, and that similar results were obtained when the substances were used in double these concentrations. With the Oxford staphylococcus the changes in opacity were similar to those shown in Fig. 1. With this organism, however, 100 $\mu\text{g.}$ cephalosporin C/ml. or 1 $\mu\text{g.}$ benzylpenicillin/ml. was necessary to delay final growth for about 35 hr. in the batch of TMB used.

Development of resistance to cephalosporin C and benzylpenicillin

Cultures obtained when *Staphylococcus aureus* strain D3R finally multiplied in aerated TMB containing either cephalosporin C or benzylpenicillin (Fig. 1) were more resistant to both antibiotics than the parent strain. Figure 2 shows the result of a typical experiment in which cocci from a culture grown in the presence of 100 μ g. cephalosporin C/ml. were inoculated into fresh medium and 200 μ g. cephalosporin C/ml. added. Heavy growth occurred almost as rapidly as from a control inoculum in medium which did not contain cephalosporin C. A similar phenomenon was observed with benzylpenicillin. For example, in one experiment the original D3R staphylococcus began to undergo the secondary multiplication after 40 hr. in the presence of 4 mg. benzylpenicillin/ml. An inoculum from the resulting culture grew up in the presence of the same concentration of benzylpenicillin after 10 hr. As measured in this way, increased resistance to cephalosporin C was accompanied by similar increased resistance to benzylpenicillin and vice versa. In serial dilution tests (read after 6 days and with the inoculum taken directly from the T-tube) the culture which had acquired resistance in the presence of benzylpenicillin was 16 times as resistant to this antibiotic as was the parent, whereas the culture which had acquired resistance in the presence of cephalosporin C was 8 times as resistant to benzylpenicillin as the parent. No evidence was obtained that an increased resistance to cephalosporin C was associated with the development of an ability to destroy this antibiotic. Samples of culture fluid taken at various times from the T-tubes were tested for cephalosporin C by the hole-plate method. Some loss of activity (at least 50%) appeared to have occurred by the time that final growth was complete. However, the grown resistant culture (Fig. 2) still contained more cephalosporin C than would have been required to bring about rapid lysis of the original culture. The resistance which developed under these conditions during the growth of a single culture was readily reversible. When cocci from the resistant culture were allowed to grow once in the absence of antibiotic, they gave rise to a new culture which was almost as sensitive as the parent strain.

Changes in resistance occurred in a similar manner when the benzylpenicillin-sensitive Oxford strain of *Staphylococcus aureus* was used; as with *S. aureus* strain D3R, there was cross-resistance to cephalosporin C and benzylpenicillin. With the Oxford staphylococcus, however, changes in resistance were accompanied by changes in morphology. The bacterial population which resulted from the final multiplication of the Oxford staphylococcus in aerated TMB consisted of varying proportions of Gram-positive cocci and Gram-negative bacilli (cf Briggs, Crawford, Abraham & Gladstone, 1957).

Experiments with bacitracin and micrococcin

Bacitracin A, a polypeptide antibiotic containing sulphur in a thiazoline ring (Lockhart, Abraham & Newton, 1955; Hausmann, Weisiger & Craig, 1955), has been reported to resemble penicillin in its effect on the incorporation of amino acids into bacterial protein (Gale & Folkes, 1953). Cross-resistance

to bacitracin and penicillin has also been reported (Paine, 1951). Micrococcin is a complex antibiotic with a high percentage of sulphur and micrococcin P, an identical or very similar substance, has been shown by Brookes, Fuller & Walker (1957) to contain the thiazole ring system. It therefore seemed worth while to compare the effects of bacitracin and micrococcin with that of benzylpenicillin on staphylococci growing in aerated TMB.

Bacitracin. When bacitracin (final concentration 154 $\mu\text{g./ml.}$) was added to a growing culture of the Oxford staphylococcus or *Staphylococcus aureus* strain D3R the changes in opacity were very similar to those shown in Fig. 1. Growth

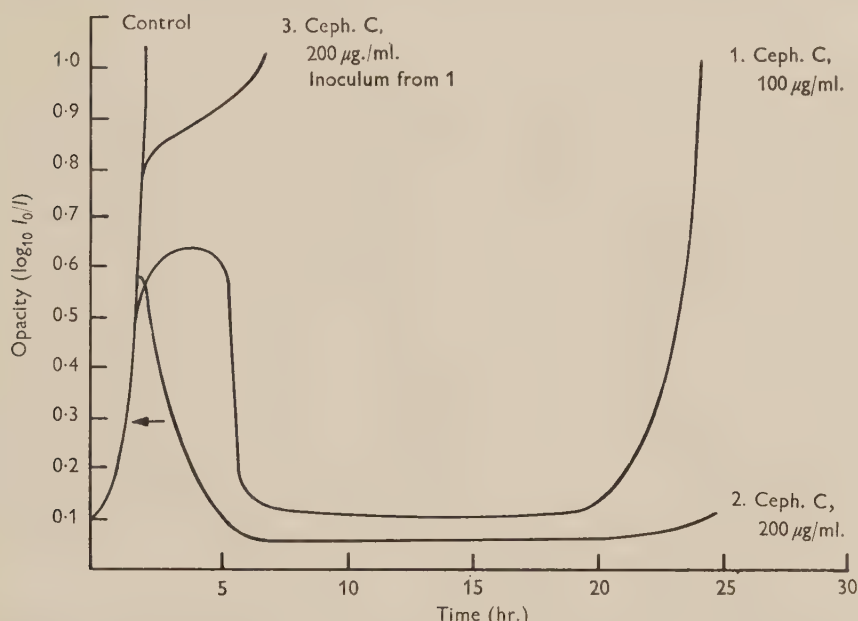


Fig. 2. Resistance acquired by *Staphylococcus aureus* strain D3R to cephalosporin C.
 ← shows the point at which the antibiotic was added.

continued rapidly for about 2 hr. and was then followed by lysis. A subsequent increase in opacity began again after 30 hr. (Oxford staphylococcus) and 20 hr. (strain D3R). In both cases the final culture consisted of a mixture of apparently normal staphylococci and Gram-negative rods in approximately equal proportions and was more resistant to bacitracin than the parent. Inocula taken from the cultures which had grown once in the presence of bacitracin (referred to as culture B₁ from the Oxford staphylococcus, and culture B₂ from strain D3R) began to undergo the secondary multiplication after 4 and 5 hr. respectively, when used in fresh medium containing the same concentration of the drug.

The increase in resistance of *Staphylococcus aureus* strain D3R to bacitracin was also found to be accompanied by an increase in resistance to cephalosporin C and benzylpenicillin. In a comparative experiment, the final increase in opacity of culture B₂ in the presence of cephalosporin C (200 $\mu\text{g./ml.}$)

occurred after 18 hr., whereas the corresponding time with strain D3R was 30 hr. In the presence of benzylpenicillin (1000 $\mu\text{g./ml.}$) the time for culture B₂ was 15 hr. and that for strain D3R was 20 hr.

Micrococcin. When micrococcin (final concentration 1 $\mu\text{g./ml.}$) was added to a growing culture of *Staphylococcus aureus* D3R, there was an immediate but relatively slight decrease in the growth rate for 3 hr., followed by a much larger decrease in the next 20 hr.; within 40 hr. the culture appeared to be almost as dense as a control culture. No detectable lysis occurred and the organisms in the final culture had the appearance of normal staphylococci.

DISCUSSION

The effect of cephalosporin C on the opacity of growing cultures of staphylococci is qualitatively very similar to that of benzylpenicillin, a fact which is consistent with the suggestion that cephalosporin C is a penicillin-like substance. The relatively low activity of cephalosporin C against penicillin-sensitive staphylococci may be associated with an inability of the organisms to concentrate this compound in the way that they concentrate benzylpenicillin. A similar suggestion was made to account for the finding that the activity of cephalosporin N against many Gram-positive organisms was much lower than that of the common penicillins (Abraham, Newton, Crawford, Burton & Hale, 1953). Pollock (1957) has shown that the relative 'affinities' of benzylpenicillin, cephalosporin N and cephalosporin C for the 'penicillin-binding component' in *Bacillus cereus* (as measured by their ability to inhibit fixation of ³⁵S from ³⁵S-labelled benzylpenicillin) bear some relationship to their relative activities as antibiotics against Gram-positive organisms. However, it is not yet possible to draw a physico-chemical picture of the processes on which these 'affinities' depend.

In contrast to the situation with the penicillin-sensitive staphylococcus, the activity of cephalosporin C in suppressing visible growth of a penicillinase-producing strain of staphylococcus for a prolonged period was much greater than that of benzylpenicillin. This fact is clearly related to the stability of cephalosporin C to the action of penicillinase. In serial dilution tests the apparent activity of benzylpenicillin was less with a large inoculum than with a small one, and it decreased greatly as the times at which the test was read was extended from 1 to 6 days. The activity of cephalosporin C, on the other hand, varied little with the size of the inoculum or with the time.

In serial dilution tests with the Oxford staphylococcus, the total activity of a mixture of cephalosporin C and benzylpenicillin was not greatly different from the sum of the activities of the separate components. This simple additive effect would be expected if both antibiotics had a similar mode of action, the difference in their separate specific activities being due to a difference in their ability to combine with the sensitive centre in the cell.

In serial dilution tests with the penicillinase-producing staphylococcus strain D3R, the activity of a mixture of cephalosporin C and benzylpenicillin was much greater than the sum of the separate activities of the components in

suppressing growth for 5–6 days. The figures given in Table 2 show that the activities of mixtures were from 7 to 10 times as great as would have been expected on the assumption that a solution to which had been added x units of cephalosporin C activity and y units of benzylpenicillin activity would contain $x + y$ units. The corresponding values obtained from an experiment in which the inoculum was 100 times as large (Table 3) vary from 4 to 7. With aerated cultures in T-tubes, in which even larger inocula were used, the activity of a mixture of the antibiotics in suppressing final growth for 1–2 days was nearly twice as great as the sum of the activities of its components (Fig. 1).

We have applied the term synergism to the increased activity of mixtures of cephalosporin C and benzylpenicillin. This word has come into use as a descriptive term for the phenomenon of two drugs together being more effective than either alone. The phenomenon may have various underlying mechanisms and it has been measured in different ways. Eagle & Saz (1955) have maintained that the term synergism should not be used when two drugs act independently on different members of a population, and that it should be restricted to those cases in which one drug augments the action of another on individual cells. It seems more convenient, however, to give it a broader meaning.

There can be little doubt that part, at least, of the synergistic action of cephalosporin C and benzylpenicillin on *Staphylococcus aureus* strain D3R is due to the fact that cephalosporin C is a competitive inhibitor of penicillinase. The two antibiotics showed no significant synergistic action on the Oxford staphylococcus, which does not produce penicillinase. Although the relative affinity of cephalosporin C and benzylpenicillin for penicillinase is known (Abraham & Newton, 1956*b*) it is not sufficient, without knowledge of other variables, for a quantitative prediction to be made of the combined effect of the two drugs in serial dilution tests. Nevertheless, some of the semi-quantitative aspects of the experimental findings seem to be understandable.

In the two serial dilution tests with *Staphylococcus aureus* strain D3R it appeared that synergism was greater with a small inoculum than with a large one and greater when the test was read after 5–6 days than when it was read after 1 or 2 days. The following considerations suggest that the synergistic effects, as measured here, will be greatest when the amount of penicillinase formed in the culture is neither very large nor very small. Suppose that growth does not occur before the concentration of benzylpenicillin has fallen to a given value. With a very big inoculum, producing much penicillinase, the destruction of benzylpenicillin will be rapid and a large amount of this drug alone will be necessary to suppress growth for a prolonged period. The inhibitory concentration of cephalosporin C alone will be relatively much smaller. The addition of subinhibitory amounts of cephalosporin C may therefore have only a minor effect on the rate of destruction of most of the benzylpenicillin, since the two compete for the penicillinase. On the other hand, if the inoculum were so small that very little penicillinase was produced, or if the tests were read as early as possible, so that the enzyme had relatively little time to act, the inhibitory concentration of benzylpenicillin alone might begin to approach

the theoretical value that it would have if no destruction by penicillinase occurred. Under such conditions the maximum synergistic effect that would be possible might not be very great. Whether a significant effect will be demonstrable under some conditions *in vivo* remains uncertain.

Both the Oxford staphylococcus and *Staphylococcus aureus* strain D3R became more resistant to cephalosporin C and benzylpenicillin when grown in aerated cultures in the presence of these antibiotics. The finding that an increase in resistance to one substance was accompanied by an increase in resistance to the other is consistent with the view that they have similar modes of action.

The Oxford staphylococcus gave rise, in the presence of cephalosporin C, benzylpenicillin, or bacitracin to a Gram-negative bacillus. Klimek, Cavallito & Bailey (1948) reported that pleomorphic Gram-negative organisms were obtained from a strain of *Staphylococcus aureus* which had been transferred 64 times in medium containing increasing amounts of penicillin. The change encountered during the present work, however, is remarkable in that it occurred during the growth of a single culture. The ability to change in this way is not confined to one strain of staphylococcus, for Gram-negative organisms were obtained with equal facility when *S. aureus* strain D3R was grown in the presence of bacitracin. Briggs *et al.* (1957) have made a further study of the bacilli formed from the Oxford staphylococcus in the presence of penicillin.

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Some Properties of Gram-negative Bacilli Obtained from a Strain of *Staphylococcus aureus* in the Presence of Benzylpenicillin

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SUMMARY: Gram-negative bacilli were obtained from the Oxford strain of *Staphylococcus aureus* in the presence of benzylpenicillin, and single organisms were isolated from these bacilli. The properties of bacilli derived from the isolates were compared with those of the parent staphylococci. The two types of organism differed strikingly in morphology, staining and various biochemical reactions. The bacilli were much more resistant to benzylpenicillin than the parent staphylococcus, but much less resistant to polymyxin. Staphylococci were recovered from all the strains of bacilli. More than half of the recovered strains resembled the original staphylococcus in all properties tested, including phage type, when first isolated. The remainder formed a somewhat heterogeneous group, differing in some properties from the parent. Later, some members of this heterogeneous group also became indistinguishable from the original staphylococcus.

Changes in the morphology of bacteria grown in the presence of subinhibitory amounts of penicillin were first observed by Gardner (1940). He found that staphylococci and streptococci became swollen, and that a number of bacilli formed long filaments. Many similar observations were made subsequently by others (see Florey *et al.* 1949). In 1944, Rake, McKee, Hamre & Houck reported that strains of *Staphylococcus aureus* which had acquired resistance to penicillin contained a proportion of organisms that did not retain the Gram stain. Klimek, Cavallito & Bailey (1948) stated that a strain of *S. aureus* which was subcultured many times in the presence of increasing amounts of penicillin and developed a very high resistance to the drug eventually became Gram-negative and bacillary in form.

In recent experiments Crawford & Abraham (1957) found that Gram-negative bacilli could be obtained from *Staphylococcus aureus* during one culture in the presence of benzylpenicillin, cephalosporin C, or bacitracin. The present paper describes a further study of the bacilli formed in the presence of benzylpenicillin. The strain of *S. aureus* used was derived from a single organism; this was grown in medium containing benzylpenicillin, and several single-cell isolations were made from the Gram-negative organisms that were obtained.

METHODS

Materials and methods of culture

Benzylpenicillin, 1650 units/mg. (Glaxo Laboratories Ltd.), was used as the crystalline sodium salt. Polymyxin B sulphate (7909 units/mg.) was kindly provided by the Wellcome Research Laboratories. The medium was in most

cases a tryptic heart digest broth (TMB) or a tryptic heart digest agar (TMA). Aerated cultures were grown in inverted T-tubes as described by Crawford & Abraham (1957). Anaerobic cultures were grown in tubes of freshly boiled TMB incubated in an anaerobic jar at 37°. Twofold serial dilution tests were used for measuring antibacterial activity.

Staphylococcus aureus (Oxford strain, NCTC 6571) was the parent organism from which all others were derived.

Methods used in the recognition of the Oxford staphylococcus

(1) *Fermentation tests.* Solutions of 1% (w/v) lactose, glucose, maltose, mannitol and sucrose in peptone water were used, with phenol red as indicator.

(2) *Growth on salt agar.* The medium used was 10% (w/v) NaCl in TMA.

(3) *Growth on penicillin agar.* Benzylpenicillin was added to TMA at almost 50° to give a final concentration of 1 µg./ml.

(4) *Production of protease.* Plates of 1% (w/v) gelatin in Difco heart infusion agar were inoculated, incubated at 37°, and then flooded with 15% (w/v) HgCl₂ in N-HCl. Protease production was shown by a clear zone round the inoculum.

(5) *Production of coagulase.* Fresh human plasma was diluted 1/10 in TMB and the resulting solution inoculated with one loopful of an overnight TMB culture, and then incubated at 37° overnight.

(6) *Production of haemolysins.* To detect α-haemolysin rabbit red blood cells were washed 3 times with saline and suspended in a volume of saline equal to the original volume of blood and plates poured with TMA mixed at c. 45° with 2.5% (v/v) of the red cell suspension. Half the plate surface was then covered with 0.1 ml. of antiserum containing 150 units of α-antitoxin (obtained from Mrs I. Batty of the Wellcome Research Laboratories, Beckenham). Cultures were inoculated on both halves of the plate and incubated at 37° in a mixture (v/v) of 20% CO₂ + 80% air. The production of a diffuse-edged lysed zone on the side of the plate not covered with antitoxin and the absence of any zone on the side covered with antitoxin indicated the presence of α-haemolysin.

δ-Haemolysin was detected in the following way. Plates were poured as described for α-haemolysin except that washed horse blood cells were used in place of rabbit cells. The plates were incubated in air at 37° after inoculation. A sharp-edged lysed zone round the colonies indicated the presence of δ-haemolysin.

(7) *Phage typing.* This was carried out by Dr R. E. O. Williams of the Staphylococcus Reference Laboratory, Colindale, using the standard procedure (Anderson & Williams, 1956).

(8) *Pathogenicity test.* Two mice (18–25 g.) were given intraperitoneally 0.5 ml. of a just visible suspension of an 18 hr. TMA culture. After 10–15 days the animals were killed and examined for lesions in the organs, from which cultures were made.

Sterility precautions and single-cell isolations. The TMB medium was sterilized by autoclaving for 20 min. at 115° in the culture vessel. All media were incubated for 2 days at 37°, under test conditions, and then for 2 or more

days at room temperature before inoculation. Benzylpenicillin was sterilized by filtration through a sintered glass bacteriological filter (maximum pore size $1.4\ \mu$). Serial subcultivation and any other manipulations involving opening the culture tubes, were done in a tissue-culture box. The box was irradiated with ultraviolet light for 15 min. before each series of manipulations. Uninoculated plates were exposed to the air inside the box during each manipulation, and then incubated at 37° for 2 days followed by 2 days at room temperature. In no case were any contaminants found. Even so, plates were never used in serial culture work.

To ensure that the cultures used began as pure lines, single-cell isolations were made by a method similar to that of de Fonbrune (1949), by means of a Singer micromanipulator (Barer & Saunders-Singer, 1951). Single organisms were placed by means of a micropipette in sterile hanging drops of TMA which were suspended in a liquid paraffin oil-cell. The micropipette was attached by pressure tubing to an 'Aglar' micrometer syringe modified to draw in and out, and both the pipette and the syringe were filled with paraffin. TMB medium for the hanging-drops was clarified by centrifuging.

Isolation of the single-cell culture (G+) from the Oxford staphylococcus. A T-tube containing 10 ml. of TMB and several sterile glass beads was inoculated with the Oxford staphylococcus and shaken for 2 hr. at 37° . This treatment gave a suspension of evenly dispersed cocci, most of which were viable. Small drops of this culture were used as reservoirs in the oil-cell from which single organisms were extracted and transferred to hanging-drops of sterile TMB. These single-seeded drops were incubated overnight at 37° , and the isolates which grew extracted with a needle, put on to TMA slopes and incubated for 18 hr. at 37° . One of these cultures was designated G+ and used as the parent strain.

RESULTS

Morphological changes in the Oxford staphylococcus grown in the presence of benzylpenicillin

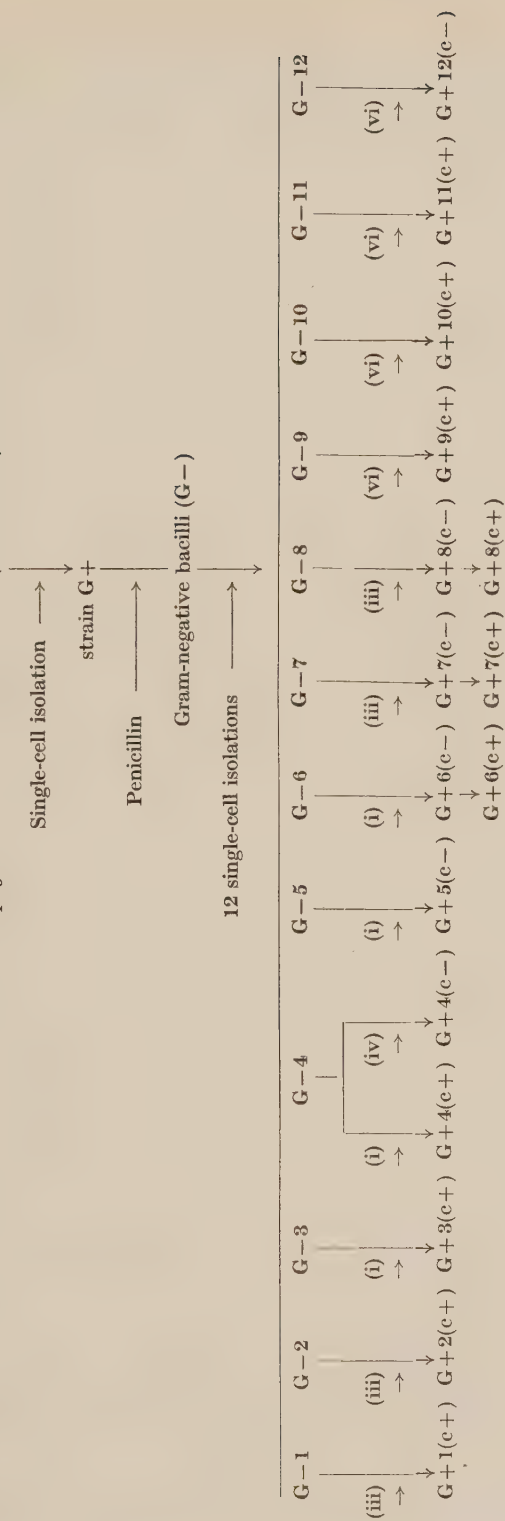
Cultures which finally developed from the Oxford staphylococcus in aerated TMB containing benzylpenicillin ($1\ \mu\text{g./ml.}$) usually consisted of a mixture of apparently normal staphylococci and Gram-negative rods. On several occasions the Gram-negative forms began to appear during the lytic phase, when the opacity was still falling (cf. Crawford & Abraham, 1957). More often, however, their appearance was delayed until the opacity was at its lowest point, or was undergoing the secondary increase.

The relative number of the two types of organism in the secondary cultures varied from one experiment to another, but in some cases the proportion of Gram-negative rods was as high as 90% (Pl. 1, fig. 1). When such mixtures were inoculated into aerated TMB not containing benzylpenicillin they yielded new cultures containing a greatly increased proportion of staphylococci (Pl. 1, fig. 2). On the other hand, when grown a second time in aerated TMB containing benzylpenicillin they developed into cultures containing mainly long Gram-negative filaments (Pl. 1, fig. 3). To ensure that this phenomenon

Table 1. Scheme showing derivation of cultures

Numbers (i)–(vi) refer to recovery method shown in text.

Staphylococcus aureus, Oxford strain (NCTC 6571)



represented a change in the staphylococcus itself further studies were made with cultures derived from single organisms.

The following work was carried out with the sterility precautions described in Methods; Table 1 outlines the relationships between the cultures obtained.

Penicillin treatment of strain G+ (the Oxford strain single-cell isolate)

Benzylpenicillin (final concentration 1 μ g./ml.) was added to an aerated culture of G+ growing in a rocking T-tube. The opacity/time curve was of the same type as those shown by Crawford & Abraham (1957, Fig. 1). The interval between the addition of benzylpenicillin and the start of the final increase in opacity was 22 hr. The resulting culture consisted of Gram-negative rods of varying lengths and a few Gram-positive cocci. The T-tube had only been opened twice since sterilization, first to add the inoculum, second to add the benzylpenicillin.

Isolations of Gram-negative rods obtained from strain G+ after penicillin treatment

Single-cell isolations of the Gram-negative rods were made from the culture described in the previous paragraph in the same manner as used to obtain strain G+. Some difficulty was experienced in this selection, as many bacilli failed to grow when seeded singly into sterile drops of TMB. However, when the primary isolate was incubated in air enriched with 5% (v/v) CO₂, the number of successful isolations was considerably increased. Twelve isolates, designated G-1 to G-12, were put on to TMA slopes and on to slopes containing 1 μ g. benzylpenicillin/ml. and incubated for 18 hr. at 37°.

Attempts to recover the original staphylococcus, strain G+, from the cultures G-1 to G-12

The following methods (i to vi) were used in attempts to recover the strain G+ from strains G-1 to G-12.

(i) The Gram-negative organisms were grown under anaerobic conditions for 2 days in TMB, after a series of eight daily subcultures in TMB.

(ii) They were subcultured daily in TMB, or on TMA slopes.

(iii) They were subcultured at two-weekly intervals, from TMA slopes which had been first incubated at 37° for 18 hr. and then left for the remainder of the 2 weeks at room temperature.

(iv) A series of eight daily subcultures in TMB or on TMA was followed by a number of subcultures in the deficient medium used by Bellamy & Klimek (1948). This medium was Seitz-filtered and incubated for 3 days before inoculation from an 18 hr. TMA slope.

(v) After two subcultures in TMB the Gram-negative organisms were subcultured twice in aerated TMB containing 10% (w/v) NaCl. The final culture was diluted (1/10) with TMB to give a salt concentration of 1%. This diluted culture was incubated at 37° for 18 hr., a loopful extracted and transferred to a TMA slope.

(vi) The Gram-negative organisms were subjected to intermittent sub-

culture on TMA slopes, for about 4 months, interspersed with periods of several weeks on the bench.

The final step in each method of isolating the Gram-positive coccus from the Gram-negative bacillus was selection on salt agar slopes, and not by plating. The possibility of an adventitious Gram-positive coccus contaminating the culture was thus reduced to negligible proportions.

The methods described above are a rather arbitrary combination of serial subculture and procedures selective for the growth of staphylococci. Nevertheless, Gram-positive cocci were recovered from eight of the twelve G— isolates (G—1 to G—8) by one or more of methods (i) to (v), of which those involving anaerobic culture and culturing at intervals of 2 weeks proved most successful, G—9 to G—12 remained unaltered under these conditions but yielded Gram-positive cocci by method (vi).

The results are shown in Table 1, in which Gram-positive strains used in further tests are designated G+1(c+) etc., where G+1(c+) was obtained from G—1 by method (iii) and was coagulase-positive, and where G+4(c+) and G+4(c—) were obtained from G—4 by methods (i) and (iv) respectively and were coagulase-positive and coagulase-negative respectively.

Comparison of strain G+ with strains G—1 to G—12 and the recovered strains G+1 (c+) to G+12 (c—)

The Gram-negative strains G—1 to G—12 were alike in all properties considered in this section, but contrasted dramatically with the parental strain G+. The staphylococci recovered from these strains may be divided into two groups, consisting of strains apparently identical with the original G+, and strains differing in some properties from strain G+.

Cultural properties in liquid media. The parent strain G+ grew vigorously in aerated TMB, well in stagnant TMB, and more slowly in anaerobic TMB. Strains G—1 to G—12 grew very rapidly in aerated TMB, but less vigorously in stagnant TMB, most of the growth forming an easily dispersed pellicle on the surface of the broth. In anaerobic TMB G—1 to G—12 grew very poorly (see attempts to recover G+ by Method (i)). The growth of all the recovered Gram-positive strains in aerated or anaerobic liquid culture was very similar to that of strain G+.

Cultural properties on solid media. Strain G+ grew well on TMA, forming regular, opaque, cream-white butyrous colonies (see white colonies in Pl. 1, fig. 4). Strains G—1 to G—12 also grew well on TMA, forming regular and somewhat translucent, smooth, slightly mucoid, yellowish white colonies (see translucent colonies—Pl. 1, figs. 4, 6). Growth on 1 µg. benzylpenicillin/ml. in TMA was good, and here the type of colony formed was variable. Young colonies obtained from Gram-negative rods which had been freshly isolated from strain G+ often had a 'speckled' appearance (Pl. 1, fig. 5). Incubation for a further 18 hr. produced a smoother growth. Sometimes the colonies on benzylpenicillin + TMA were smooth, especially when the strain had been subcultured several times after the original isolation, but such colonies were occasionally sectoried by wedges of the 'speckled' growth (Pl. 2, fig. 7).

The colonies of the recovered strains, G+1(c+), G+2(c+), G+3(c+), G+4(c+), G+4(c-), G+9(c+), G+10(c+) and G+11(c+), when grown on TMA resembled those of strain G+ in colour and opacity, whereas those of strains G+5(c-), G+7(c-), G+8(c-) and G+12(c-) differed in being more opaque and whiter, and strain G+6(c-) had a slightly pinkish tinge.

Morphological properties. The G+ organisms were those of a typical staphylococcus with cocci regular in shape and size arranged in bunches (in hanging-drop cultures) and staining Gram-positive (see cocci in Pl. 1, fig. 2). In marked contrast, strains G-1 to G-12 showed organisms varying from very long rods to very short oval forms in the same culture. The rods were usually continuous along their length, but were occasionally in chains of shorter bacilli. They were very pleomorphic, non-motile, predominantly Gram-negative but with occasional thickenings, lumps and club-shaped distortions of Gram-positive material. The morphology of these organisms altered when they were grown in different media, and for different periods of incubation. In aerated TMB growth of strains G-1 to G-12 was made up of consistently Gram-negative rods, medium to short in length and fairly uniform in width (see bacilli in Pl. 1, fig. 2). When 1 μ g. benzylpenicillin/ml. was added, very long rods were formed, again fairly uniform in width (see filaments in Pl. 1, fig. 3). In stagnant TMB medium a pellicle was formed which consisted of rods of varied shape and length, often with Gram-positive lumps and patches. After several subcultures in stagnant TMB medium, the rods became shorter and many stained Gram-positive. Eventually, after a series of subcultures it was possible to recover Gram-positive cocci from some of the single-cell isolates (see attempts to recover strain G+ by Method (ii)). In anaerobic conditions the rods did not grow as such, but as distorted Gram-positive cocco-bacilli (see attempts to recover strain G+ by Method (i)). On solid media colonies were made up of Gram-negative rods showing a wide range of forms. These varied from very long, exceedingly pleomorphic filaments, when grown on 1 μ g. benzylpenicillin/ml. in TMA (Pl. 2, fig. 8), to very short Gram-negative cocco-bacilli (Pl. 2, fig. 12), when subcultured on TMA without penicillin. There were intermediate types with different proportions of long, medium and short rods (Pl. 2, figs. 9-11). The proportion of short cocco-bacilli increased with longer incubation and with more subcultures without penicillin. Over-all, a well-aerated medium containing penicillin seemed to provide the best conditions for the most extravagant forms of long bacillus.

The cocci of all the recovered G+ strains resembled those of the original strain G+ in size, regularity of shape, arrangement and Gram-staining.

Sensitivity to benzylpenicillin and polymyxin B. Dilution tests were used to determine the sensitivity of some of the organisms to benzylpenicillin and to polymyxin B. The concentrations of these antibiotics which were inhibitory to the growth of representative strains for 1 and 4 days respectively are shown in Table 2. It will be seen that strains G+, G+2(c+), G+3(c+) and G+6(c-) were of the same order of sensitivity to benzylpenicillin, while strains G-3 and G-12 were extremely resistant. Moreover, with the Gram-negative strains the end-point was not clear-cut, and there were traces of growth even at

concentrations as high as 10,000 $\mu\text{g.}$ benzylpenicillin/ml. In contrast, the Gram-negative organisms were much more sensitive to polymyxin than the Gram-positive ones.

Table 2. *Sensitivity to penicillin and polymyxin of staphylococci and Gram-negative variants*

The organisms are named according to the code given in Table 1.

Organism	Concentration of penicillin ($\mu\text{g./ml.}$) inhibitory for		Concentration of polymyxin B ($\mu\text{g./ml.}$) inhibitory for	
	1 day	4 days	1 day	4 days
G +	0.03	0.06	50	100
G + 2(c +)	0.015	0.015	50	100
G + 3(c +)	0.06	0.12	50	50
G + 4(c -)	.	.	50	100
G + 6(c -)	0.03	0.06	.	.
G - 3	100	1000	0.78	0.78
G - 11	.	.	0.24	0.24
G - 12	50	1000	0.20	0.20

Biochemical properties. The results of Tests 1-6, described in Methods, are shown in Table 3. Strain G + fermented the sugars tested, was able to grow on 10 % NaCl agar, and produced protease, coagulase and α -haemolysin. The G - isolates differed from strain G + in every one of these tests. Of the recovered strains, G + 1(c +) to G + 4(c +) and G + 9(c +) to G + 11(c +) had test reactions identical with those of the original strain G +, whereas the remainder did not produce coagulase and differed from strain G + in several other tests. Strains G + 4(c -) and G + 12(c -) were unable to ferment mannitol and did not produce protease or α -haemolysin. The remaining four strains were also unable to ferment mannitol, and produced in addition a different haemolysin, namely δ -haemolysin. Of these, only strain G + 5(c -) produced protease, and only strain G + 8(c -) was unable to ferment lactose.

Phage typing. Dr R. E. O. Williams reported as follows: 'The parent strain, G +, was susceptible to phages, 7, 52, 52A, 79 and 80 when used 1000 times stronger than the routine test dilution; all other tests were therefore made with the strong phage filtrates. Seven of the recovered staphylococci had phage sensitivity patterns that were practically identical with the parent strain (Table 3); these were the seven that produced coagulase and α -haemolysin. The other six strains were untypable. None of the cultures that consisted entirely of Gram-negative bacilli was lysed by the phages. The supernatant fluids of cultures of six representative strains (G + 1(c +), G + 2(c +), G + 4(c +), G + 4(c -), G - 6, G - 7) were tested for the presence of phage using five strains of phage group 1 as indicators. The G + (c +) strains all carried a phage lysing the same indicator strain, which had, in routine typing, the phage sensitivity pattern 52A/79. No phage could be found in the supernatants of the untypable strains.'

Pathogenicity tests. These tests were carried out with strains G +, G - 2, G - 3, G - 6, G - 7, G - 11, G - 12, G + 2(c +), G + 3(c +), G + 6(c -) and G + 7(c -). No

Table 3. *Summary of the properties of the Oxford staphylococcus, Gram-negative variants, and recovered staphylococci*

Organism	Tests*										
	1					2	3	4	5	6	7
	Fermentations of sugars					Growth on salt agar	Growth on penicillin agar	Protease	Coagulase	Haemolysins	
	Lactose	Glucose	Mannitol	Sucrose						α	δ
Colony colour on TMA	Gram reaction										Phage type
I. Parent coccus	+	+	+	+	+	+	+	+	+	+	52/52 A/79/80/7 +
II. Bacilli from I	-	+	-	-	-	-	+	-	-	-	Untypable
G-12											
G+1(c+)											
G+2(c+)											
G+3(c+)											
G+4(c+)											
G+9(c+)											
G+10(c+)											
G+11(c+)											
III. Coagulase-positive cocci from II											
G+4(c-)											
G+5(c-)											
G+6(c-)											
IV. Coagulase-negative cocci from II											
G+7(c-)											
G+8(c-)											
G+12(c-)											
V. Coagulase-positive cocci from IV											
G+6(c+)											
G+7(c+)											
G+8(c+)											

* Tests 1-7 described in Methods. In test 1, + = acid, no gas; - = growth but no acid; sl + = slow, + after 48 hr. In tests 2-6, + = presence and - = absence of the property under test.

deaths occurred in the mice injected and they were killed after 13 days. No lesions were found with the G- strains, or with either of the two coagulase-negative strains G+6(c-) and G+7(c-). The coagulase-positive strains G+, G+2(c+) and G+3(c+) produced large abscesses in the body wall, from which the same strain was recovered. No lesions were found elsewhere.

*Change of coagulase-negative (c-) strains of staphylococci
to parent type*

Subsequently, after about 4 months of subculture and storage, it was found that three of the coagulase-negative strains (G+6(c-), G+7(c-), and G+8(c-)) had reverted to coagulase-positive strains with the same phage type as the parent strain G+. The change in properties can be seen in Table 3. The nature of this reversion is being investigated.

Thus, Gram-positive cocci were recovered from each of the twelve Gram-negative single-cell bacillary isolates. Ten of these recovered strains were identical with the original strain G+, seven being identical when first isolated and three passing through an intermediate stage in which they differed from the parent in several characters.

*Other properties of pure-line staphylococci and
Gram-negative variants*

Growth rates. The rate of growth of strains G+, G-7 and G+3(c+) was compared in aerated TMB by measuring changes in opacity, the initial opacity being about 0.2 on the scale used. No significant differences in growth rate were observed. A mixture of strains G-7 and G+ and another of strains G-7 and G+3(c+) (containing about 50% of each strain) grew at the same rate as the single strains alone. No change in the composition of the mixtures was observed during growth.

Production of penicillinase. A 5-day culture of the Gram-negative strain G-3 in TMB was kept at 37° with benzylpenicillin (1 mg./ml.) for 16 hr. A solution of benzylpenicillin in water (1 mg./ml.) was used as a control. No difference was detected in the final activity of the two solutions. In a similar experiment, in which the penicillinase-producing strain D3R was used, complete loss of penicillin occurred in the presence of a 5-day culture.

DISCUSSION

The present work, in which single cocci of the Oxford staphylococcus were used and exceptional precautions were taken to avoid contamination, leaves no doubt that Gram-negative filaments were really formed from staphylococci during the growth of a single culture in the presence of benzylpenicillin and that they were able to revert to the parent strain. The reason why they were obtained so much more rapidly than in the experiments with penicillin previously described by others is uncertain, but it may be connected with the use of large inocula and aerated cultures. In serial dilution tests, which were carried out in stagnant media, cultures which grew up in the presence of the

antibiotics always consisted of Gram-positive cocci. Klimek *et al.* (1948) reported that Gram-negative organisms obtained from *Staphylococcus aureus* in the presence of penicillin were unable to grow anaerobically. These organisms had also lost the ability to ferment a number of simple carbohydrates, although they were still able to ferment glucose; Gale & Rodwell (1948, 1949) showed that their Gram-negative organisms derived from staphylococci had lost the power of concentrating free glutamic acid within the cells, and had become able to synthesize all their amino acid requirements from ammonia and glucose in the presence of thiamine.

The mixed cultures obtained when the Oxford staphylococcus was grown once in aerated medium containing cephalosporin C, benzylpenicillin, or bacitracin reverted to cultures consisting almost entirely of normal staphylococci during one subculture in aerated medium devoid of antibiotic (Crawford & Abraham, 1957). Our pure-line Gram-negative variants did not undergo noticeable reversion under these conditions, but they reverted to staphylococci in old colonies on solid media or when subcultured under anaerobic conditions. Klimek *et al.* (1948) and Bellamy & Klimek (1948) noted that staphylococci with a moderate degree of resistance to penicillin reverted readily to a sensitive strain, but that cultures which had a very high resistance were more stable. It seems that the Gram-negative variants which are first formed are less stable than their progeny. However, it is not certain whether the reversion of the early mixed cultures is due to a change of Gram-negative organisms to staphylococci or to an ability of the staphylococci to grow faster than these Gram-negative organisms in the absence of antibiotic. Although the growth rate of a pure-line Gram-negative variant did not differ greatly from that of the original staphylococcus in aerated TMB, this was not necessarily the case with the variants that were first produced.

The pure-line Gram-negative variants differed strikingly from staphylococci, not only in morphology and in resistance to certain antibiotics, but also in a number of biochemical properties. They failed to ferment five different sugars, did not liquefy gelatin, produced neither coagulase nor haemolysins, and were resistant to all phages used to type coagulase-positive staphylococci. Some of the staphylococci recovered from these variants were identical with the original Oxford strain in all the properties tested, including phage type. Others, however, had failed to regain the ability to form coagulase and α -haemolysin, to ferment mannitol or to liquefy gelatin, and could not be typed by phage. Eventually, some of the latter reverted to the parent type. The formation of the Gram-negative variants may also be a multi-stage process. It is well known, for example, that staphylococci can acquire a high resistance to benzylpenicillin without losing their ability to retain the Gram stain (Klimek *et al.* 1948; Kawamata & Shigeto, 1956). It has also been reported that a penicillinase-producing strain of *Staphylococcus aureus* can give rise, in the presence of penicillin, to cocci which no longer produce penicillinase, are coagulase-negative and fail to ferment mannitol or liquefy gelatin (Vourek, 1952).

Very little information is available, from these experiments, about the

mechanisms by which such striking changes in the staphylococcus occur. During the initial period of lysis in aerated cultures most of the original staphylococci are certainly killed, the relative decrease in opacity being much less than the decrease in number of viable organisms. Gram-negative forms were sometimes observed during lysis, although the full secondary growth did not occur until much later. Although selection clearly plays a role in the development of the cultures of Gram-negative organisms and in the production, from the latter, of cultures of staphylococci, the hypothesis that all the changes are initiated by spontaneous, independent, mutations is not free from difficulty. A considerable number of different mutations, each of some selective value in the same environment, would seem to be required and the reversal of all of them would be necessary to account for the recovery of what appears to be the original staphylococcus. It is difficult to believe that changes in all the properties tested are associated with differences in growth rate in the presence and absence of benzylpenicillin. Induced mutations, akin to that which has been reported to be responsible, under some conditions, for the development of resistance to streptomycin (Szybalski, 1955), would also need to be readily reversible. These difficulties are not greatly minimized by the fact that some of the cultures of recovered staphylococci were at one stage distinguishable from the parent strain. The hypothesis that a single genetic change is responsible, directly or indirectly, for changes in a number of different properties, is complicated by the fact that the recovered staphylococci were heterogeneous.

It seems possible, therefore, that some, at least, of the changes observed in the present work occurred without loss of genetic continuity and resulted from the effect of the environment on extranuclear structures. The formation of the Gram-negative bacilli might involve adaptive changes in slowly growing organisms during the lysis of organisms which were growing more rapidly. The cytochemical studies of Boivin, Tulasne, Vendrely & Minck (1947), which indicated that penicillin interfered with the synthesis of ribonucleic acid and hindered cytoplasmic division without affecting nuclear division, and later work, which has suggested that the primary effect of penicillin is on the synthesis or maintenance of a component of the cell wall (Mitchell, 1957; Cooper, 1956; Lederberg, 1956), may be relevant in this connexion. The finding that a Gram-negative variant was much more sensitive to polymyxin than the original or recovered staphylococcus supports the view that a major change had occurred in the cell wall. Newton (1956) has obtained evidence that the bactericidal activity of polymyxin is a consequence of its ability to damage a membrane which underlies the cell wall and controls osmotic equilibrium. He has suggested that polymyxin is relatively ineffective against most Gram-positive bacteria because it cannot readily penetrate their cell walls and reach this sensitive membrane.

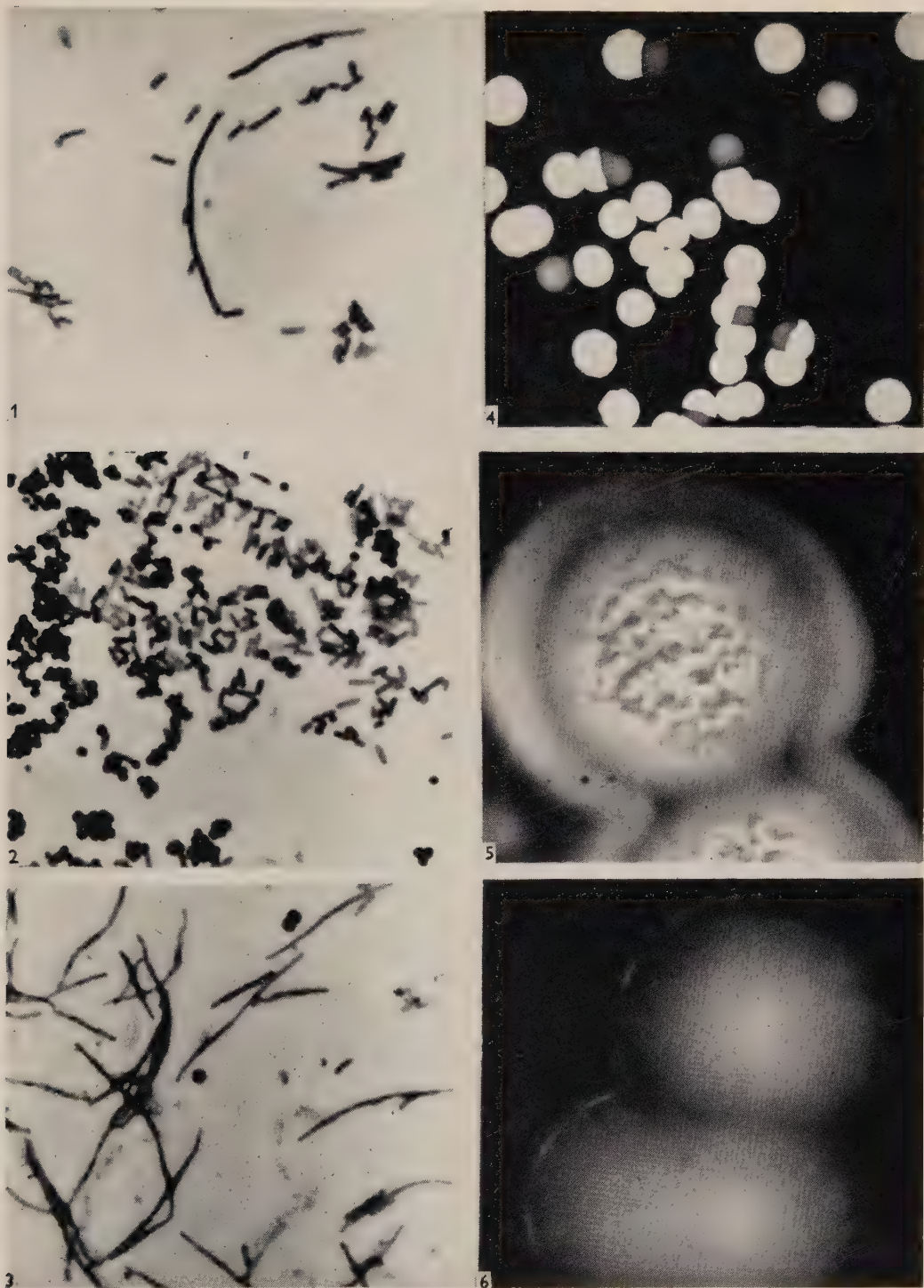
During the formation of the Gram-negative bacillary forms the production of active phage is suppressed. The phage is still absent when the bacillary form changes to a coagulase-negative staphylococcus, but reappears when it reverts to a coagulase-positive staphylococcus. The capacity to form phage has been

reported to control the expression of certain inheritable characters in bacteria—for example, the production of diphtheria toxin (Freeman, 1951), and antibiotics have been shown to alter the character of lysogenic diphtheria bacilli by removal of carried phage (Hewitt, 1954). It is therefore conceivable that the inability of some of the coagulase-negative staphylococci to produce phage is linked with other properties in which these organisms differ from those of the parent strain. This hypothesis, however, would not account for all the facts, because some of the staphylococci which failed to carry phage were not identical in other respects.

We wish to thank Dr R. E. O. Williams for his active co-operation in carrying out investigations into the phage type and lysogenicity of our strains. Our thanks are due to Miss Mary Read and Mrs P. Curtiss for technical assistance and to Mr F. Bradley for photographs. One of us (S.B.) is indebted to the Medical Research Council for a personal grant.

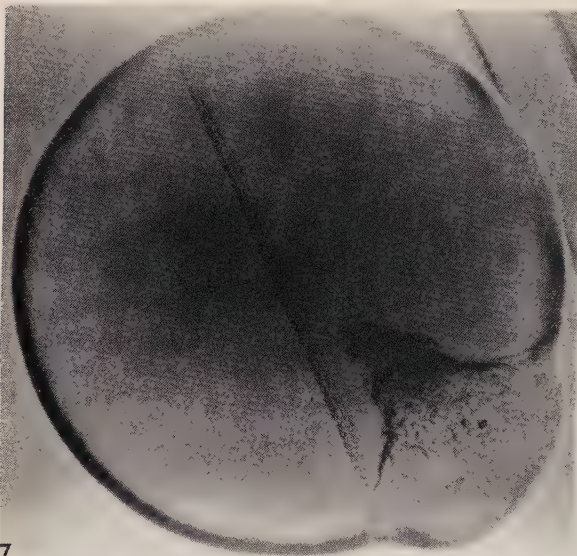
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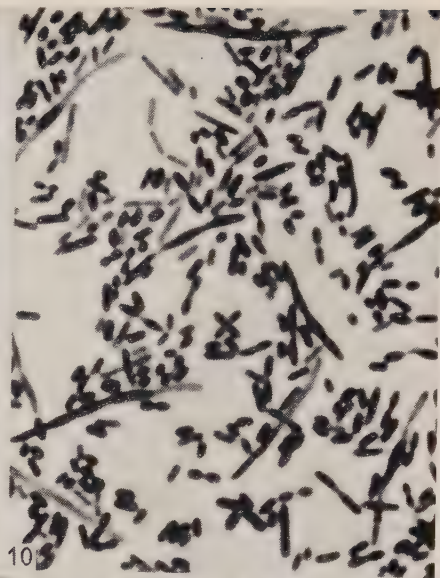


S. BRIGGS AND OTHERS—GRAM-NEGATIVE BACILLI FROM STAPHYLOCOCCI. PLATE 1

(Facing p. 626)



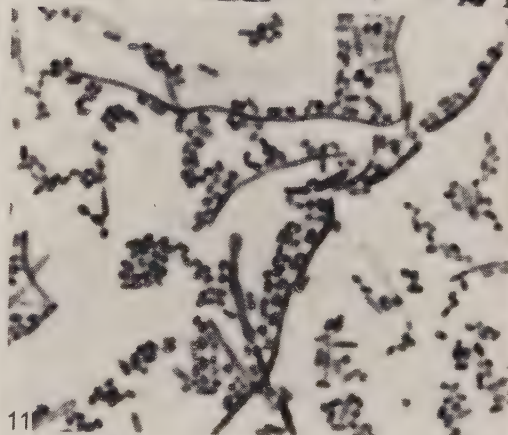
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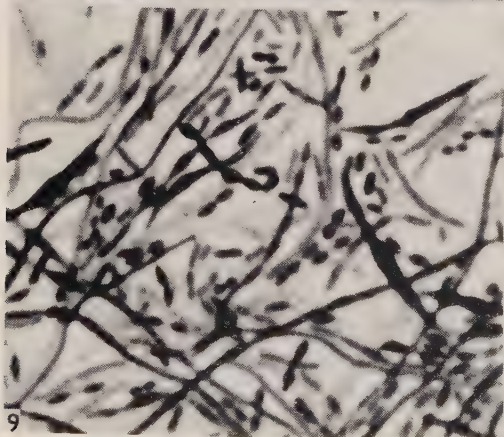
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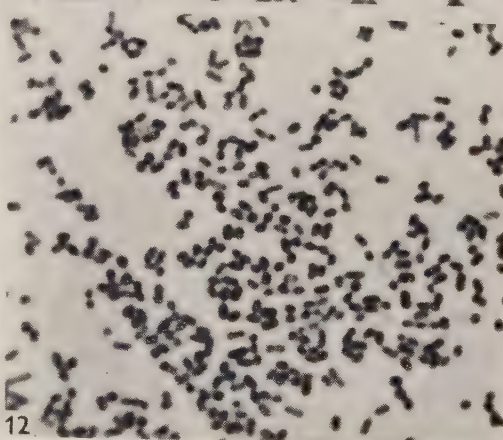
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EXPLANATION OF PLATES

PLATE 1

Variants of *Staphylococcus aureus* (single-cell isolate G+, from Oxford strain NCTC 6571) after treatment with penicillin (1 μ g./ml.). All microscopical preparations were Gram stained (magnification $\times 1500$).

- Fig. 1. The first growth of Gram-negative bacillary variants in aerated TMB containing penicillin which had been inoculated with *S. aureus*. A few cocci (not shown in the preparation) were still present.
- Fig. 2. Subculture of the growth shown in fig. 1, into aerated TMB without penicillin, showing short Gram-negative bacilli and an increased proportion of Gram-positive cocci.
- Fig. 3. Subculture of the growth shown in fig. 1, into aerated TMB containing penicillin. The culture is composed mainly of Gram-negative filaments with a few enlarged cocci.
- Fig. 4. Colonies of staphylococci (white) and bacillary variants (translucent) on TMA ($\times 2$).
- Fig. 5. Colonial appearance of bacilli grown on TMA containing 1 μ g./ml. penicillin for 2 days at 37°, showing the 'speckled' appearance of growth on the first day and a ring of smooth growth which appeared during the second day's incubation ($\times 30$).
- Fig. 6. Colonial appearance of bacilli grown on TMA for 2 days at 37° ($\times 30$).

PLATE 2

- Fig. 7. 'Smooth' colony of bacilli with a sector of 'speckled' growth, on TMA with penicillin, incubated 2 days at 37°, then left 4 days at room temperature ($\times 30$).
- Fig. 8. Gram-negative filaments from the 'speckled' portion of a colony similar to that shown in Fig. 5 but after incubation for 1 day only.
- Fig. 9. Gram-negative filaments derived from a sector of the colony shown in fig. 7. The sector was transferred to TMA containing penicillin and the plate incubated for 2 days. The Gram-negative filaments were obtained from one of the resulting colonies.
- Fig. 10. Bacilli derived from a sector of the colony shown in fig. 7. In this case part of the sector was transferred to TMA containing no penicillin.
- Fig. 11. Bacilli derived from the 'smooth' part of the colony shown in fig. 7. A portion of the colony was plated on TMA containing penicillin and the plate incubated for 1 day. The bacilli were obtained from the resulting colonies.
- Fig. 12. Appearance of the Gram-negative variants after 5 subcultures on TMA without penicillin. The organisms are Gram-negative cocco-bacilli.

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Carbohydrate Metabolism of *Paramecium aurelia*, Variety 4, Stock 47.8 (Sensitive)

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SUMMARY: Homogenates of *Paramecium aurelia*, var. 4, stock 47.8 (sensitive), grown in axenic culture contain enzymes of the glycolytic, the citric acid cycle, and the pentosephosphate pathway. The presence of the following enzymes has been demonstrated: hexokinase, phosphoglucumutase, phosphohexoisomerase, aldolase, triosephosphate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, pentosephosphate isomerase, citric acid oxidase, isocitric acid dehydrogenase, α -ketoglutaric acid dehydrogenase, succinic acid oxidase, malic acid dehydrogenase, glutamic acid dehydrogenase, fumarase, and acid phosphatase. The presence of phosphorylase, transaldolase and transketolase is indicated.

Studies on the metabolism of ciliates have been notably deficient in the investigation of the presence of the individual enzymes of the metabolic sequences. Most studies have been confined to adding compounds to media in which the protozoans were to be grown. Increased multiplication or an increased oxygen uptake measured in the conventional Warburg apparatus was taken to indicate that the compounds under investigation were metabolites. Humphrey & Humphrey (1948) found that succinic acid would cause a twofold increase in the respiration of *Paramecium caudatum*. In studies on the utilization of carbohydrates by the same organism Geddes & Humphrey (1951) established that glycogen, glucose, fructose, glucose-1-phosphate, glucose-6-phosphate and fructose-1:6-diphosphate were utilized. A few studies on the presence of enzymes of the electron transport system in *Paramecium*, notably cytochrome oxidase, have been made by Clark (1945) and Boell (1945), while Sato & Tamiya (1937) demonstrated the presence of cytochromes c, a and b in *Paramecium*. Simonsen & van Wagtendonk (1952) suggested that the respiration of killer stocks of *P. aurelia*, which, unlike the respiration of sensitive stocks, is not inhibited by KCN, may be almost entirely mediated by flavin systems. In a later study (Simonsen & van Wagtendonk, 1956), the same authors reported the presence in killer cells of an active succinoxidase system which is absent, or present only in small concentrations in the sensitives. The metabolism of *Tetrahymena pyriformis* has been extensively studied and the presence of many enzymes involved in the various phases of carbohydrate metabolism has been established. This work has been reviewed by Seaman (1955).

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While many enzymic studies on the metabolism of *Paramecium aurelia* can be carried out in bacterized media, it is imperative for certain experiments of long duration involving homogenates that these be free from contaminants. With the advent of an axenic medium for *P. aurelia* (van Wagtendonk, Conner, Miller & Rao, 1953; Miller & van Wagtendonk, 1956), an investigation of its carbohydrate metabolism became possible without the danger of interference by other organisms.

METHODS

Paramecium aurelia, var. 4, stock 47.8 (sensitive), was grown axenically in the medium given in Table 1 or in the medium described by Vloedman (1955). Large cultures were derived from cultures in test tubes by transferring to

Table 1. *Composition of axenic medium for Paramecium aurelia*

Component	Concn. ($\mu\text{g./ml.}$)	Component	Concn. ($\mu\text{g./ml.}$)
Vitamins		Amino acids	
Pantothenic acid	2.0	L-Arginine	200
Nicotinamide	2.0	L-Histidine	100
Pyridoxal	2.0	DL-Isoleucine	300
Biotin	0.03	L-Leucine	300
Riboflavin	2.0	L-Lysine	250
Folic acid	1.0	DL-Methionine	300
Thiamine	6.0	L-Phenylalanine	150
		DL-Serine	400
Salts		DL-Threonine	300
Sodium ethylenedinitrilotetraacetate	10.0	L-Tryptophan	100
MgSO ₄ .7H ₂ O	50.0	L-Tyrosine	75
Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O	12.5	DL-Valine	150
MnCl ₂ .4H ₂ O	0.25	L-Alanine	50
ZnCl ₂	0.03	L-Aspartic acid	100
CaCl ₂ .2H ₂ O	25.0	Glycine	50
CuCl ₂ .2H ₂ O	2.5	L-Glutamic acid	150
FeCl ₃ .6H ₂ O	0.63	L-Proline	100
K ₂ HPO ₄	500.0	Yeast fractions (mg./ml.)	
KH ₂ PO ₄	500.0	Precipitate*	3.0
Sodium acetate	500.0		
Stigmasterol	0.2		

* The precipitate was obtained from a water extract of yeast (Conner & van Wagtendonk, 1953) by addition of an equal volume of acetone and centrifugation of the mixture (Conner & van Wagtendonk, unpublished).

increasingly larger volumes, up to 500 ml. in a 3 l. Erlenmeyer flask. At maximum population density (5000–10,000 organisms/ml.), the organisms were harvested by filtering the culture through a sterile Berkefeld filter. Further concentration was obtained by electromigration through a sterile salt solution according to the procedure described by van Wagtendonk, Simonsen & Zill (1952), followed by a final centrifugation at 850 *g* for 10 min. The resulting pellet was suspended in a suitable amount of buffer, homogenized with the apparatus described by Katzberg (1950), a hand homogenizer of the Potter-Elvehjem type, or by exposure to ultra-sound in a 10 kc. oscillator (water

cooled Raytheon), and made up to the desired volume with the same buffer. Cell-free extracts were prepared by centrifugation of the homogenates at 24,000 *g* in an angle centrifuge for 30 min. The protein content of the homogenates and the cell-free extracts was determined according to the method of Gornall, Bardawill & David (1949). In some instances the N content of the homogenates was determined with the micro-Kjeldahl method using the digestion mixture of Brüell, Holter, Linderstrøm-Lang & Rozits (1947).

Reduction of 2,3,5-triphenyltetrazolium chloride (TTC) was used for the determination of dehydrogenases. The determinations were carried out in conical graduated centrifuge tubes (15 ml.). The reaction mixture consisted of 0.5 ml. substrate at a suitable concentration; 0.1 ml. 0.001 % (w/v) diphosphopyridine nucleotide (DPN, 65 % pure, Sigma); 0.1 ml. 0.05 M-cysteine; 0.1 ml. 0.1 M-MgCl₂; 0.5 ml. 0.001 % (w/v) TTC; 0.5 ml. homogenate and 0.1 M-tris-(hydroxymethyl)-amino-methane (Tris) buffer to a final volume of 2.5 ml., depending upon the determination. The reaction mixture was incubated at 37° for various lengths of time and the reaction stopped by the addition of 1 ml. 10 % (w/v) trichloroacetic acid (TCA). The resulting precipitate, consisting of protein and reduced and unreduced TTC, was spun down at 850 *g* in a refrigerated centrifuge. The supernatant fluid was poured off, and the pellet extracted twice with 2 ml. acetone. One ml. 10 % TCA was added to the second acetone extract. (The extraction of the precipitate with chloroform-acetone as recommended by Green, Mii, Mahler & Bock (1954) was not applicable here, due to the large amounts of protein present in the precipitate.) Additional acetone was added to bring the final volume to 7 ml. The optical density was read on a Klett-Summerson colorimeter with filter no. 54 (540 m μ). The readings were compared with those obtained from a standard curve, which was prepared by the reduction of TTC with sodium sulphide according to Black & Speer (1953). A few crystals of sodium sulphide were added to known amounts of TTC (10–100 μ g.) dissolved in 1 ml. distilled water. The reaction was allowed to proceed for 1 hr., 1 ml. 10 % TCA solution was then added and the mixture brought to a final volume of 7 ml. with acetone. The optical densities were determined on the Klett-Summerson colorimeter using filter no. 54.

Determinations of other enzymic activities were made according to published procedures, and the compositions of the reaction mixtures are given in the table headings and the figure legends.

Demonstration of the presence of enzymic pathways

Phosphorylase. Homogenates of *Paramecium aurelia* stock 47.8 contain an active phosphorylase, the presence of which was demonstrated by the method described by Whelan (1955), except that glycogen was substituted for starch. The results are given in Table 2.

The liberation of orthophosphate in the absence of added glycogen is not due to the action of acid phosphatase, because in *Paramecium aurelia* this enzyme has a low activity at pH 6.0 (Fig. 9). Apparently enough stored

glycogen is present in *P. aurelia* to allow the reaction to proceed in the absence of added glycogen.

Hexokinase. This enzyme was determined by following the reduction of triphosphopyridine nucleotide (TPN) in a reaction mixture containing glucose, a cell-free extract of *Paramecium aurelia* and an added excess of glucose-6-phosphate dehydrogenase, according to the method of Wood & Schwerdt (1953). A cell-free extract was prepared by centrifugation of a homogenate at 24,000 g for 15 min. at 4° in an angle centrifuge. The results are given in Fig. 1.

Table 2. *Phosphorylase in homogenates of Paramecium aurelia, var. 4, stock 47.8*

The complete reaction mixture contained 0.2 ml. 4% (w/v) glycogen solution, 100 μ mole glucose-1-phosphate, 4.0 ml. homogenate containing 26.4 mg. protein and 0.1 M-citrate-NaOH buffer to make a total volume of 6 ml. pH 6.0, temp. 38°.

Reaction mixture	μ mole phosphorus liberated/mg. protein					
	Minutes					
	0	5	10	30	60	120
Complete	0	0.003	0.012	0.036	0.079	0.136
Minus glycogen	0.002	0.007	0.011	0.036	0.073	0.130
Minus glucose-1-phosphate	0	0	0	0	0	0
and glycogen						
Minus homogenate	0	0	0	0	0	0

Phosphoglucumutase. The presence of this enzyme in homogenates is indicated by the fact that glucose-1-phosphate could also act as substrate for the dehydrogenation by glucose-6-phosphate dehydrogenase (Fig. 1). The transformation of glucose-1-phosphate to glucose-6-phosphate was followed by the decrease in acid-labile phosphate (7 min. hydrolysis in 1.0 N-HCl at 100°) in a test system described by Najjar (1948) (Fig. 2).

Phosphohexoisomerase. The removal of fructose-6-phosphate from a reaction mixture and the formation of fructose reactive material from glucose-6-phosphate provided evidence for the presence of phosphohexoisomerase in homogenates (Sable & Calkins, 1953) (Fig. 3).

Aldolase. The presence of this enzyme was demonstrated by incubating a homogenate with the Mg-salt of fructose-1:6-diphosphate, using hydrazine as the trapping agent for the trioses formed (Sibley & Lehninger, 1949; Beck, 1955), and measuring the triosehydrazone by its conversion to the chromogen of the 2,4-dinitrophenylhydrazine derivative. The trapped triosehydrazone was also measured by determining the amount of alkali-labile phosphate formed (Meyerhof & Lohmann, 1934). The results are given in Table 3.

Triosephosphate dehydrogenase was determined in a reaction mixture containing Mg-fructose-1:6-diphosphate, homogenate and TTC. The reduction of TTC indicated the presence of a triosephosphate dehydrogenase (Table 4). The addition of hydrazine blocked the reduction of TTC, presumably by trapping the triose intermediates, and the addition of iodoacetic acid (IOA) prevented the same reaction presumably by inhibiting the triosephosphate dehydrogenase itself.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase react specifically with DPN in *Paramecium aurelia* and the change in absorption at 340 m μ . with time is a measure of the reaction. An active cell extract was prepared by twice-repeated freezing and thawing of a homogenate. The latter was prepared in a Potter-Elvehjem type homogenizer. The mixture was centrifuged for 30 min. at 850 g to prepare a cell-free extract. The reaction mixture of Wood & Schwerdt (1953) was used. The results are given in Fig. 4.

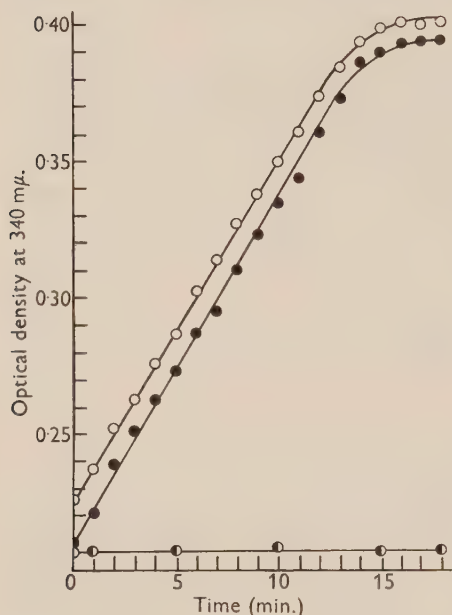


Fig. 1

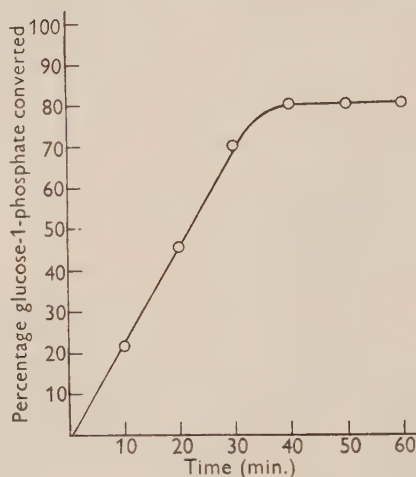


Fig. 2

Fig. 1. Demonstration of the presence of hexokinase in cell-free extracts of *Paramecium aurelia*. The reaction mixture contained: 0.3 ml. cell-free extract (1.8 mg. protein), 5 μ mole $MgCl_2$, 50 μ g. TPN (Sigma, 80 % pure), 3 mg. glucose-6-phosphate dehydrogenase (Sigma, 0.15 Kornberg units/mg.), 3.3 μ mole substrate, 0.25M-glycylglycine buffer (pH 7.4) to a final volume of 3 ml. The change in optical density at 25° was determined with a Beckman DU spectrophotometer at 340 m μ . in cuvettes with 1 cm. light path. ○—○, glucose-1-phosphate; ●—●, glucose; ○—○, no substrate.

Fig. 2. Phosphoglucomutase activity of homogenates of *Paramecium aurelia*. The complete test systems consisted of: 3 ml. homogenate (19.2 mg. protein), 50 μ mole Tris buffer (pH 7.5), 10 μ mole $MgCl_2$, 10 μ mole glucose-1-phosphate (Schwarz Laboratories), 50 μ mole cysteine. Total volume 6 ml., temp. 32°. Reactions stopped at 10 min. intervals with 5 ml. of 6% (w/v) perchloric acid and the reaction mixture hydrolysed for 7 min. in 1N-HCl in a boiling water bath. Liberated inorganic phosphate was determined according to Fiske & SubbaRow (1925). No easily hydrolysable phosphate was detectable in the absence of substrate.

Pentose metabolism. When ribose-5-phosphate was incubated in the presence of a homogenate of *Paramecium aurelia*, TTC and DPN, reduction of TTC was observed indicating the oxidation of ribose-5-phosphate. Addition of hydrazine prevented the reduction of TTC (Table 5). The course of this oxidation

could be further analysed with the same reaction mixture using the colour reactions described by Dische & Borenfreund (1951) and by Mejbaum (1939). An increase in absorption over the range from 450 to 700 m μ . of the chromogen formed in the Dische-Borenfreund reaction indicated the formation of keto-pentose, suggesting the presence of pentosephosphate isomerase (Fig. 5). This

Table 3. *The formation of chromogen by trapped triosehydrazone and alkali labile phosphate formed by the action of aldolase*

The reaction mixtures contained: 0.5 ml. homogenate (7.1 mg. protein), 12.5 μ mole Mg-fructose-1:6-diphosphate, 100 μ mole Tris buffer (pH 8.5), 140 μ mole hydrazine sulphate (pH 8.6), final volume 2.5 ml., temp. 37°. The reactions were stopped at various time intervals with 2 ml. 10 % (w/v) trichloroacetic acid and the chromogen developed in a 1 ml. sample. The optical density was measured in the Klett-Summerson colorimeter at 540 m μ . (filter no. 54) against the reaction mixture without substrate as a blank. Alkali labile phosphate was determined on a separate 1 ml. sample.

Time (min.)	μ mole alkali labile phosphate liberated/mg. protein		increase in optical density of chromogen at 540 m μ .	
	Experimental	Endogenous	Experimental	Endogenous
10	0.019	—	0.038	—
20	0.039	—	0.164	—
30	0.065	—	0.300	—
40	0.093	—	0.390	—
50	0.099	—	0.480	—
60	0.124	0.028	0.600	—

Table 4. *Triosephosphate dehydrogenase in homogenate of Paramecium aurelia*

The complete system contained: 0.5 ml. homogenate (10 mg. of protein), 25 μ mole Mg-fructose-1:6-diphosphate, 5 μ mole cysteine, 0.1 μ mole DPN, 20 μ mole K₂HPO₄, 1.5 μ mole TTC, 70 μ mole Tris buffer (pH 7.5), 2.5 μ mole MgCl₂, 50 μ mole hydrazine or 2 μ mole iodoacetic acid (IOA) were included where indicated—total volume 2.0 ml., temp. 38°.

	μ mole TTC reduced		
	30 min.	60 min.	90 min.
Complete system	0.037	0.054	0.088
Complete system plus IOA	—	0.010	0.007
Complete system plus hydrazine	—	0.019	0.008
Endogenous	—	0.017	0.024

Table 5. *Oxidation of ribose-5-phosphate by homogenate of Paramecium aurelia*

The reaction mixtures consisted of 14.7 μ mole ribose-5-phosphate, 1.5 μ mole TTC, 0.1 μ mole DPN, 10 μ mole MgCl₂, 20 μ mole K₂HPO₄, homogenate containing 15.5 mg. protein, 0.1 M-Tris buffer (pH 7.6) to 3.0 ml. The reactions were stopped at 15 min. intervals by adding 1 ml. 10 % (w/v) TCA.

	μ mole TTC reduced/mg. protein				
	Minutes				
	15	30	45	60	75
Complete	0.026	0.046	0.054	0.068	0.073
— Ribose-5-phosphate	0.020	—	—	—	0.023
+ Hydrazine	—	—	—	—	0.018

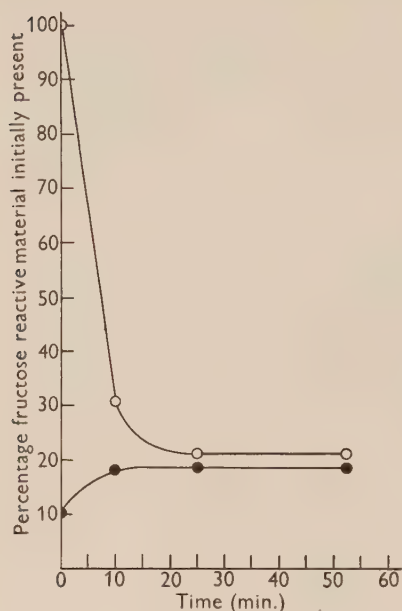


Fig. 3

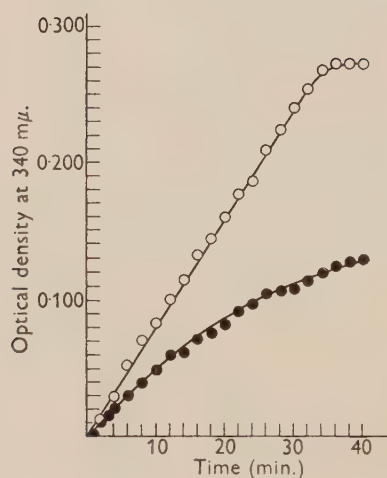
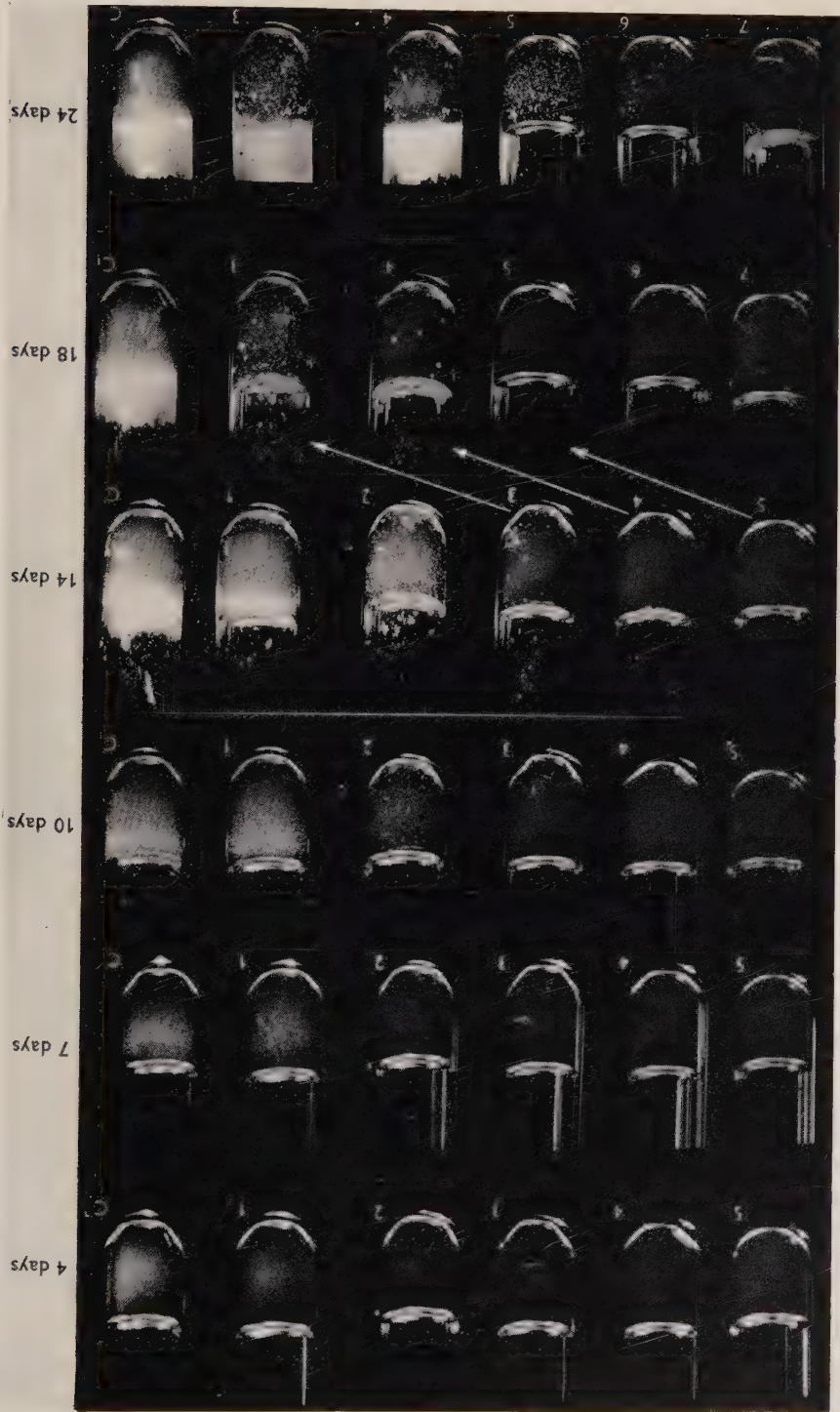


Fig. 4

Fig. 3. Demonstration of the presence of phosphohexoisomerase in homogenates of *Parametium aurelia*. The reaction mixture contained 3 ml. homogenate (18.2 mg. protein), 10 μ mole substrate (glucose-6-phosphate or fructose-6-phosphate), 50 μ mole Tris buffer (pH 7.5), 10 μ mole *p*-chloromercuribenzoate. Total volume 5 ml., temp. 32°. Volumes of 1 ml. were removed at intervals, and the reaction stopped by the addition of 5 ml. 6% (w/v) perchloric acid. Fructose reactive material in the supernatant after centrifugation was determined according to Roe (1934). In these analyses account was taken of the fact that fructose-6-phosphate gives considerably less colour than an equimolar amount of free fructose. The factor 0.75 was used to correct for this difference. ○—○, fructose-6-phosphate; ●—●, glucose-6-phosphate.

Fig. 4. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase in *Parametium aurelia* extracts. The medium contained: 3.3 μ mole substrate, 25 μ g. DPN (Sigma, 65% pure), 5 μ mole $MgCl_2$ and 0.25 ml. *P. aurelia* extract (1 mg. protein). Tris buffer (0.1 M, pH 7.8) to 0.5 ml., temp. 27°. The optical density was measured in the Beckman DU spectrophotometer using cells with a light path of 1 cm. The readings were made against the above reaction mixture from which the substrate was omitted. There was no endogenous reduction of DPN. ○—○, glucose-6-phosphate; ●—●, 6-phosphogluconate.

is further corroborated by an analysis of the orcinol spectra of the reaction mixture at 0 hr. and at 2 hr. (Fig. 6). Concomitant with the decrease of the aldopentose peak at 670 $m\mu$, an increase in the ketopentose peak at 540 $m\mu$. is noted. At the same time aldopentose reactive material disappears from the reaction mixture (Table 6). According to Horecker and co-workers (Horecker, Smyrniotis & Seegmiller, 1951; Seegmiller & Horecker, 1952; Horecker, Smyrniotis & Klenow, 1953), the ratio D_{540}/D_{670} for aldopentose is 0.19 and for ribulose 0.90. Similarly, the ratio D_{670}/D_{580} for sedoheptulose is 0.29 and for pentose 3.78. The course of the change of these two ratios is given in Table 7, the results being consistent with the formation of ketopentose and, in addition,



R. KNOX & R. WOODROFFE—DRUG SENSITIVITY OF *M. TUBERCULOSIS*. PLATE I

(Facing p. 659)

Table 6. Utilization of aldopentose reactive material by homogenates of *Paramecium aurelia*

The reaction mixture was identical with that of Fig. 5.

	μ mole of aldopentose		
	0 min.	5 min.	120 min.
Experimental	18.43	17.21	11.83
Endogenous	2.63	2.98	4.09
Aldopentose remaining	15.80	14.23	7.74

Table 7. Change in D_{540}/D_{670} and D_{670}/D_{580} ratios during incubation of ribose-5-phosphate with homogenate of *Paramecium aurelia*

The reaction mixture was identical with that in Fig. 7.

Ratio	Minutes				
	0	10	30	60	120
D_{540}/D_{670}	0.27	0.32	0.36	0.61	0.63
D_{670}/D_{580}	4.51	4.17	3.61	2.11	1.92

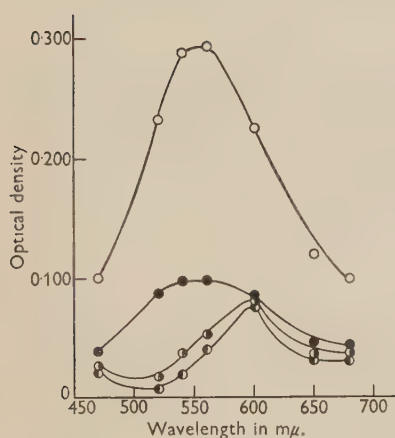


Fig. 5

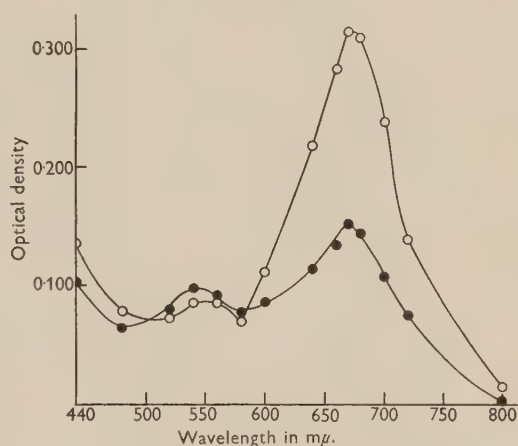


Fig. 6

Fig. 5. Formation of ketopentoses as indicated by the change in absorption spectra. The reaction mixture consisted of 15.80 μ mole ribose-5-phosphate, 375 μ mole Tris buffer (pH 7.0) and 27.4 mg. homogenate protein in a total volume of 4 ml., temp. 27°. 0.5 ml. samples were removed at various time intervals and added to 4.5 ml. 10% TCA (w/v). 1 ml. samples were used for the Dische-Borenfreund reaction and 0.2 ml. samples for the orcinol reaction. Absorption spectra were measured in the Beckman DU spectrophotometer in a cell with a 1 cm. light path. ●—●, experimental zero time; ○—○, experimental 120 min.; ●—●, endogenous zero time; ○—○, endogenous 120 min.

Fig. 6. Formation of ketopentoses by homogenate of *Paramecium aurelia*. The curves represent the change in absorption spectra over the range of 440–800 m μ . of the chromogens formed in the orcinol reaction. The reaction mixture was identical with that of Fig. 5. ○—○, zero time; ●—●, 120 min.

of heptose, as probable intermediates in the oxidation of pentosephosphate. This probability is strengthened by the observation that *Paramecium* homogenates form pentoses from sedoheptulose. A pentose peak appears in the orcinol spectrum of the reaction mixture (Fig. 7), while at the same time the concentration of sedoheptulose decreases (Fig. 8).

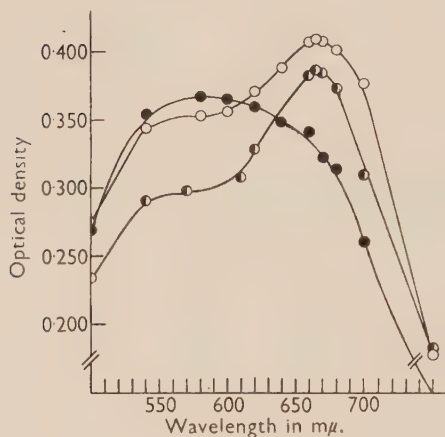


Fig. 7

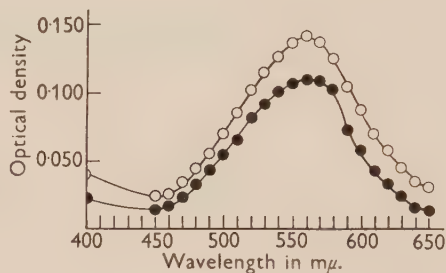


Fig. 8

Fig. 7. Formation of pentoses from sedoheptulose by homogenate of *Paramecium aurelia*. The curves represent the absorption spectra of the chromogen formed in the orcinol reaction by the sedoheptulose and pentoses. The reaction mixture consisted of 9 μ mole sedoheptulose [kindly furnished by Dr B. L. Horecker as the Ba-salt (78 % pure)], 390 μ mole Tris buffer (pH 7.6), 0.1 mg. cocarboxylase complex (Difco), 2 ml. homogenate (41 mg. protein). Final volume 5 ml., temp. 37°. 1 ml. samples were removed at various times and added to 4 ml. 10 % (w/v) TCA. 0.5 ml. samples were used for the Dische-Borenfreund and orcinol reactions. ●—●, zero time; ○—○, 60 min.; ○—○, 120 min.

Fig. 8. Disappearance of sedoheptulose. The curves represent the absorption by the chromogen of sedoheptulose formed in the Dische-Borenfreund reaction at 0 and at 120 min. The composition of the reaction medium was identical with that of Fig. 7. ○—○, 0 min.; ●—●, 120 min.

Enzymes of the citric acid cycle. Of the tricarboxylic acid cycle acids, citric acid, isocitric acid, α -ketoglutaric acid, succinic acid, malic acid and glutamic acid can be oxidized by homogenates of *Paramecium aurelia* in the presence of DPN or TPN and TTC. The results are given in Table 8.

Since TTC is a sluggish electron acceptor, the oxidation of succinic acid and of α -ketoglutaric acid by homogenates was measured manometrically following the method of Eisenberg (1953). It can be seen from Table 9 that the oxidation of these two acids proceeds faster when other electron acceptors are used.

Fumarase. The presence of this enzyme was demonstrated by measuring the increase in optical density at 240 m μ . (Racker, 1950) due to the appearance of the double bond of fumaric acid in a reaction mixture containing malic acid and a homogenate. The fumarase activity was mostly found in the non-sedimentable fraction (at 10,000 g for 30 min.) of the homogenate (Table 10). This

Table 8. *Oxidation of citric acid cycle intermediates by Paramecium homogenates*

The reaction mixtures contained 10 μ mole substrate, 1 μ mole DPN or 0.3 μ mole TPN, 5 μ mole cysteine, 10 μ mole MgCl_2 , 10 μ mole TTC, 150 μ mole Tris buffer (pH 7.4), 1 ml. *Paramecium* homogenate (9.7 mg. protein). Total volume 2 ml.; gas phase air; temp. 37°. Identical reaction mixtures were treated as described in the text at zero time, 30 and 60 min.

Substrate	μ mole TTC reduced	
	30 min.	60 min.
Citric acid	0.08	0.29
Isocitric acid	0.43	0.46
α -Ketoglutaric acid	0.004	0.008
Succinic acid	0.03	0.05
Malic acid	0.19	0.31
Glutamic acid	0.11	0.19
Endogenous	0.002	0.006

Table 9. *Oxidation of α -ketoglutarate and succinate by homogenate of Paramecium aurelia*

The reaction medium for the determination of the oxidation of α -ketoglutarate by homogenates contained 10 μ mole substrate, 50 μ mole Tris buffer (pH 7.3), 5 μ mole MgCl_2 , 0.5 mg. DPN, 10 μ mole methylene blue, 3.5 Lipmann units of CoA and 0.5 ml. homogenate (4.6 mg. protein). The reaction medium for the determination of the oxidation of succinate contained 10 μ mole substrate, 50 μ mole phosphate buffer (pH 7.3), 0.6 μ mole cytochrome *c* (Sigma), and 0.5 ml. homogenate (4.6 mg. protein). For both determinations: final volume 1 ml.; gas phase air; temp. 32°; 0.2 ml. 10 % KOH (w/v) in the centre well.

Substrate	μ mole O_2 /mg. protein	
	30 min.	60 min.
α -Ketoglutarate	3.7	6.2
Succinate	1.1	1.8
Endogenous	0.1	0.2

Table 10. *Distribution of fumarase activity in homogenates of Paramecium aurelia*

The reaction mixture consisted of 25 μ mole malate (pH 7.4), 125 μ mole Tris buffer (pH 7.5), homogenate preparation 0.1 to 0.5 ml. as required. Total volume 3 ml. The increase in optical density at 240 $m\mu$. was measured with the Beckman DU spectrophotometer. The original homogenate was centrifuged in a Sorvall SS-1 angle centrifuge at 10,000 *g* for $\frac{1}{2}$ hr. at room temperature. A measured volume of the supernatant was used for the determination of the fumarase activity. The sediment was taken up in 10 ml. Tris buffer (0.1 M, pH 7.4) and the fumarase activity was determined on a measured volume.

Fraction	Total volume (ml.)	Protein/ml. (mg.)	Total protein (mg.)	Units*/ml.	Total units	Specific activity†
Original homogenate	6	10.81	64.86	133‡	798	12.3
Sediment	10	5.59	55.9	14	140	2.5
Supernatant	3.1	2.86	8.9	43	133.3	14.97

* One unit equals the amount of enzyme causing an increase in optical density of 0.001 per min.

† Specific activity equals number of units per mg. of protein.

‡ Activity determined on a 1:10 (v/v) dilution of the whole homogenate.

distribution is similar to that found in human liver (Shepherd, Li, Mason & Ziffren, 1955).

Phosphatase activity was measured at both acid and alkaline pH values using Na-phenolphthalein phosphate as substrate (Huggins & Talalay, 1945). Phosphatase activity could only be detected in the acid range, with an optimum activity at pH 4.5 (Fig. 9).

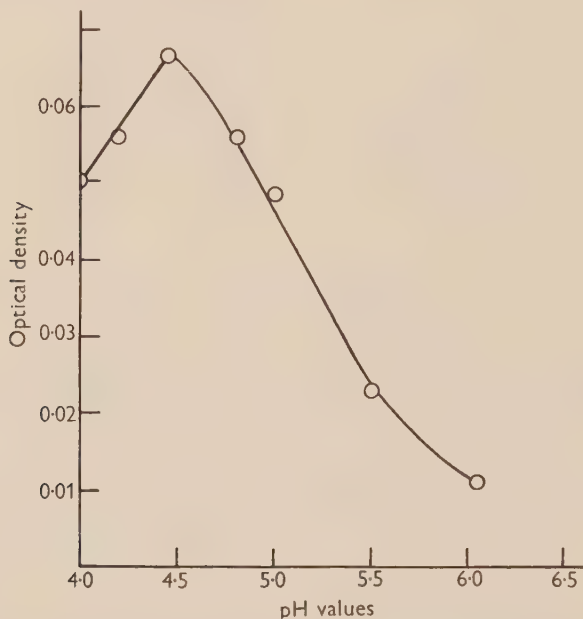


Fig. 9. Effect of pH value on activity of acid phosphatase in homogenate of *Paramecium aurelia*. The reaction mixtures consisted of: 5 μ mole Na-phenolphthalein phosphate, 0.3 mg. homogenate protein, 0.1 M-Na acetate buffer to 5 ml. pH adjusted to desired value with 0.1 N-HCl. Temp. 38°. The reactions were stopped with 1 ml. 1 N-NaOH after 60 min., and the liberated phenolphthalein measured in the Klett-Summerson colorimeter using filter no. 54.

DISCUSSION

The quantitative aspects have not been stressed in this exploratory study. The picture that emerges from this investigation is that of a conventional metabolic pattern. *Paramecium aurelia*, var. 4, stock 47.8 (sensitive) can metabolize carbohydrates via the three main routes, the glycolytic, the citric acid cycle and the pentosephosphate pathways. Which of the two oxidative schemes, the citric acid cycle or the pentosephosphate pathway, is preferentially utilized has not been determined. An attractive postulate, which is at present under investigation, is that the three pathways are of equal importance for the organism, the glycolytic and citric acid pathways providing the required energy, and the pentosephosphate pathway serving mainly to supply the organism with pentoses. The pentose demand can conceivably be very high in view of the large amount of genetic material carried by *P. aurelia*, specifically in the macronucleus (Moses, 1950). The presence of pentosephosphate iso-

merase, transaldolase and transketolase has not been unequivocally proven, but the circumstantial evidence for the participation of these enzymes in the pentosephosphate pathway is strong. The reduction of TTC with ribose-5-phosphate as substrate and the formation of ketopentose reactive material from added ribose-5-phosphate are indicative of the presence of pentosephosphate isomerase. Similarly, the formation of pentoses from sedoheptulose-7-phosphate is evidence for the establishment of an equilibrium in which transketolase and transaldolase are participating enzymes.

Levine (1955) reported some enzymic studies carried out with *Paramecium aurelia*, var. 4, stock 51.8 (sensitive) and *P. aurelia*, var. 8, stock 135.15 (sensitive). His results are in several aspects at variance with those reported here. Levine was not able to demonstrate the first two steps in the pentosephosphate pathway, the oxidation of glucose-6-phosphate and the oxidation of 6-phosphogluconate. It was suggested that this apparent absence of the oxidative pathway for the utilization of glucose-6-phosphate was due to the availability of pentoses from the 'rather opulent diet the bacteria provide'. If this were the case, the presence of the pentosephosphate pathway in axenically cultured *P. aurelia* could be considered to be an adaptation to the environment. Another explanation, e.g. that there is a difference in enzymic make-up between different stocks, is plausible, although Levine was unable to find such differences between his two stocks.

Seaman (1955) states: 'Although purity of the culture and the availability of chemically defined media are prerequisites for growth and nutrition experiments, these considerations are not of prime importance in purely metabolic studies. If a protozoan can be cultured whatever the complexity of the medium—even with the presence of bacterial contaminants—it is eligible for investigation.' Our studies, however, again emphasize the importance of axenic cultures for biochemical and physiological studies of protozoa, although this emphasis might be tempered by the question: 'How many of the findings, biochemical, nutritional, or even genetic, can be ascribed to adaptation to environmental changes from a natural habitat, via dixenic to axenic cultures?' Only further investigations might provide the answer.

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Growth of *Trichophyton mentagrophytes* and *Trichophyton rubrum* in Increased Carbon Dioxide Tensions

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SUMMARY: The formation of macrospores in normally mycelial strains of *Trichophyton mentagrophytes* and *T. rubrum* was induced by growing the cultures in a gas phase containing air + 12-24% (v/v) CO₂. When conidial transfers were made from strains which had thus been induced to form macrospores and were incubated in normal air, the subsequent growth was mycelial and downy. One strain of each fungus, normally conidial and granular, formed arthrospores under increased CO₂.

The bases for genus and species differentiation of the superficial dermatophytes are shape and size of macroconidia and gross cultural characteristics (Emmons, 1934). Identification of these *Fungi imperfecti* is complicated by loss of the ability to form macroconidia and variation in cultural morphology; most of the dermatophytes exist in two morphological types, conidial and mycelial (Wilhelm, 1947). When these fungi are first isolated from lesions on to Sabouraud's glucose agar, they usually grow as the conidial, macroconidia-forming, type. Upon repeated transfer, however, they often lose the ability to sporulate and continue growth as the mycelial type. Reversion of the mycelial to the conidial form is very uncommon. Investigations in this laboratory have shown that non-sporulating strains of *Trichophyton mentagrophytes* and *T. rubrum* can be induced to form macroconidia, thus reverting to the conidial type, by incubation in a gas phase containing concentrations of carbon dioxide above that of normal air (c. 0.03%, v/v).

METHODS

Cultures. The cultures employed in this study included 5 strains of *Trichophyton mentagrophytes* (408, My-874-55, A-538, X-15, X-10) and 6 strains of *T. rubrum* (T-3, X-14, My-38-56, X-2, My-274-56, My-640-55). Of these, only strains X-10 and X-14 were the conidial type and formed typical macroconidia and microconidia on Sabouraud's glucose agar when incubated in normal air; the other strains formed microconidia but no macroconidia under these conditions. Strain 408 formed vegetative mycelium without differentiated structures. All cultures were maintained as stocks on slopes of Sabouraud's glucose agar (Difco). After inoculation, slope cultures were incubated at 30° for 1 week then sealed with tinfoil and wax stored at 5° until used.

Inoculum. The inoculum was prepared by dispersing the growth from a stock slope with 10 ml. sterile water in a Waring blender for 1 min. One drop of this suspension was inoculated on to the centre of each of a series of

Sabouraud's glucose agar plates. Eight or more of these plates were incubated in each concentration of carbon dioxide.

CO₂ concentration. Carbon dioxide concentration was adjusted, after a set of plates had been sealed in a Brewer's anaerobic jar, by drawing a vacuum of approximately 30 mm. Hg in excess of the desired concentration of CO₂. The desired amount of CO₂ was run in from a cylinder and was measured by noting the fall in negative pressure on a manometer, then the valve of the jar was momentarily opened to return the internal pressure of the jar to atmospheric. Control plates were incubated in laboratory air.

All cultures were incubated at 30° and the CO₂ concentrations were re-adjusted daily. During the period of incubation, 2 plates from each concentration were removed at 2- to 3-day intervals for microscopic examination of the mycelium. At the end of 7 or more days of incubation, all the remaining plates were removed and examined macroscopically and microscopically.

RESULTS

Macroconidia formation by 3 out of 4 mycelial strains of *Trichophyton mentagrophytes* and 5 out of 5 mycelial strains of *T. rubrum* was induced by incubation at increased concentrations of CO₂; Table 1 indicates the response to CO₂ of all the cultures of definite history that were tested. However, this response to CO₂ was discovered with a stock strain of *T. mentagrophytes* and 2 similar strains of *T. rubrum* which are not included in this discussion because of their unknown backgrounds. Pl. 1, fig. 1, shows typical mycelial growth of *T. rubrum* T-3; Pl. 1, fig. 2, shows the conidial growth of the same strain under 16% (v/v) CO₂; this profuse formation of macroconidia is typical of most of the other strains when incubated under CO₂. *T. mentagrophytes* 408, which did not respond to increased CO₂, was not typical of other downy strains; it was completely vegetative at all times, producing neither macro- nor microconidia. The conidial strains *T. rubrum* X-14 and *T. mentagrophytes* X-10 formed arthrospores exclusively at CO₂ concentrations of 16% or more; Pl. 1, fig. 3, shows arthrospore formation by *T. rubrum* X-14. To determine that the increased concentration of CO₂ and not the decreased concentration of oxygen was inducing macroconidia formation, *T. mentagrophytes* strain A-538 was incubated in an atmosphere containing 20% (v/v) more nitrogen than normal. Under these conditions, macroconidia were not produced and no changes in morphology were apparent.

As macroconidia formation increased with increasing CO₂ concentrations, the macroscopic appearance of mycelial strains changed gradually from downy to granular; Pl. 1, fig. 4, shows the increased granulation and pigmentation of *Trichophyton rubrum* T-3 as the CO₂ concentration was increased. As the concentration of carbon dioxide in the atmosphere was increased, the diameters of the colonies decreased somewhat (Table 2).

At the concentrations of CO₂ shown in Table 1 the dermatophytes fell into four groups with regard to pigment formation: (i) the intensity of pigment increased stepwise as CO₂ concentration was increased from 0 to 24%

Table 1. *The effect of increased concentrations of carbon dioxide on macroconidia formation by strains of Trichophyton mentagrophytes and T. rubrum.*

Three plates were examined for each concentration.

Organisms	Increased CO ₂ in gas phase (% v/v)					Period of incubation (days)
	0	12	16	20	24	
	Effect on growth					
<i>T. mentagrophytes</i>						
408	—	—	—	—	—	7
My-874-55	—	—	+	++	++	7
A-538	—	nr	+++	+++	+++	7
X-15	—	+++	+++	+++	+++	7
X-10	++	nr	arth.	arth.	arth.	7
<i>T. rubrum</i>						
T-3	—	+++	+++	nr	nr	11
My-38-56	—	—	—	++	++	9
X-2	—	—	—	++	+++	9
My-274-56	—	++	+++	+++	+++	7
My-640-55	—	—	++	+++	+++	8
X-14	+++	+	arth.	arth.	arth.	9

— = no macroconidia; + = occasional macroconidia; ++ = abundant macroconidia; +++ = profuse macroconidia. arth. = arthrospore formation only. nr = not run at this concentration.

Table 2. *Effect of increased carbon dioxide concentrations on the colony diameter of Trichophyton mentagrophytes and T. rubrum*

Three plates were averaged for each concentration.

Organisms	CO ₂ in gas phase (% v/v)					Period of incubation (days)
	0	12	16	20	24	
	Colony diameter (mm.)					
<i>T. mentagrophytes</i>						
408	54	50	49	47	46	7
My-874-55	52	48	45	39	36	7
A-538	40	nr	30	26	25	7
X-15	47	27	26	27	25	7
X-10	47	nr	34	32	30	7
<i>T. rubrum</i>						
X-14	25	21	20	18	18	9
My-38-56	34	28	25	23	21	9
X-2	35	33	30	26	24	9
My-274-56	25	23	18	17	16	7

nr = not run at this concentration.

(strains T-3, My-874-55, A-538); (ii) the intensity of pigment decreased step-wise as CO₂ concentration was increased from 0 to 24 % (strains 408, My-38-56, X-10, X-14, My-640-55); (iii) the intensity of pigment increased from 0 to 12 % CO₂, then decreased from 12 to 24 % CO₂ (strain X-15); (iv) the intensity of pigment decreased from 0 to 12 % CO₂, then increased from 12 to 24 % CO₂ (strains My-274-56, X-2). There seemed to be no consistent relationship

between the two species or strains within a species regarding the relation of pigmentation and CO_2 concentration.

Conidial subcultures from mycelial strains which were induced to form macroconidia under increased CO_2 were made at the end of the incubation periods. After incubation for a week in air at 30° , these subcultures again had the typical downy gross morphology and no macroconidia were found upon microscopic examination.

DISCUSSION

In this laboratory the terms 'downy' and 'granular' are used for macroscopic descriptions, and 'conidial' and 'mycelial' are used for microscopic descriptions. The difference between granular and downy is not clear-cut; one strain may appear more downy or granular than another and intermediates exist between both extremes. Conidial strains (X-10, X-14) form macroconidia on slopes of Sabouraud's glucose agar in normal atmospheric conditions of incubation; mycelial strains (My-874-55, A-538, My-38-56, X-2, My-274-56, My-640-55, X-15, T-3) do not, both conidial and mycelial strains form microconidia. A granular strain is usually of the conidial type and a downy strain is usually in the mycelial type. However, strains which appear downy may form a few macroconidia and strains which appear granular because of intensive microconidia formation may form no macroconidia. Because of this colonial variation, any cultural condition resulting in more consistent macroscopic and microscopic morphology is noteworthy.

The reason for the change from the conidial type to the mycelial type in the dermatophytes is not known, although Wilhelm (1947) and Robbins & McVeigh (1949) suggested that a mutation at some point in the life cycle of the fungus was involved. The observations made here, namely, the rapid change from the mycelial to the conidial type by increased concentrations of carbon dioxide and the immediate return to the mycelial type upon incubation in laboratory air, seems to argue against mutation.

Whether carbon dioxide acts as a physical or chemical agent in producing these morphological changes is not at present known. Macrospore formation under increased CO_2 concentration may be associated with the metabolism of purines and pyrimidines or tricarboxylic cycle components since CO_2 is readily fixed into these compounds by many micro-organisms. However, it is not apparent why some strains form macroconidia readily on Sabouraud's glucose agar in normal atmospheric conditions, while others must be induced by increased concentrations of carbon dioxide to form these spores and lose the ability immediately upon transfer to normal atmosphere.

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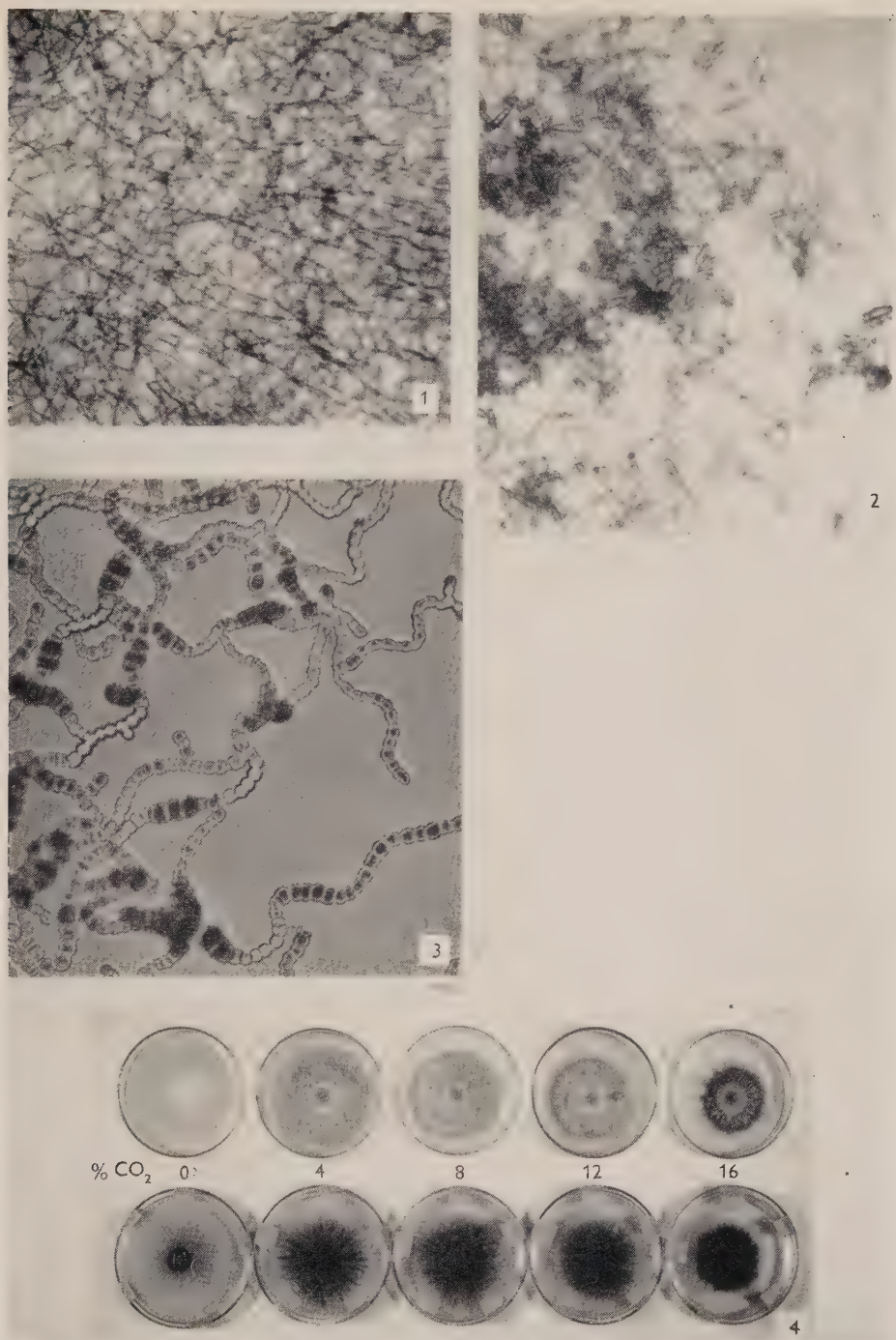
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EXPLANATION OF PLATE

- Fig. 1. Mycelium of *Trichophyton rubrum* T-3 incubated in air; a few microconidia can be seen ($\times 280$).
- Fig. 2. Mycelium of *T. rubrum* T-3 showing profuse macroconidia formation after incubation for 11 days in air + 16 % (v/v) CO₂ ($\times 280$).
- Fig. 3. Arthrospore formation by granular *T. mentagrophytes* X-10 incubated in air + 16 % CO₂ ($\times 280$).
- Fig. 4. Giant colonies of *T. rubrum* T-3 incubated 9 days at different concentrations of CO₂. Increased granulation and pigment formation is apparent.

(Received 3 December 1956)



B. CHIN & S. G. KNIGHT —CARBON DIOXIDE AND THE GROWTH OF DERMATOPHYTES.
PLATE 1

(Facing p. 646)

KNOX, R. & WOODROFFE, R. (1957). *J. gen. Microbiol.* **16**, 647-659

Semi-solid Agar Media for Rapid Drug Sensitivity Tests on Cultures of *Mycobacterium tuberculosis*

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SUMMARY: Semi-solid agar media prepared as previously described (Knox, 1955; Knox, Swait & Woodroffe, 1956) were found especially useful for studying the sensitivity of cultures of *Mycobacterium tuberculosis* to isoniazid, streptomycin and *p*-aminosalicylic acid (PAS). Kirchner, Fisher and Dubos media were compared both as semi-solid and as liquid media. In the semi-solid media when large inocula of *M. tuberculosis* H37 Rv strain were used, results could be read in 2 days with a hand-lens and were usually easily read with the naked eye in 3-5 days. Results were easier to read and interpret in semi-solid than in the corresponding liquid media both because of the sharpness of the initial end-point defining the minimum inhibitory concentration of the drug (M.I.C.), and because the later growth of numerous or isolated colonies gave a measure, not obtainable with liquid media, of the variability within a culture. Such presumptively resistant colonies appeared most frequently with isoniazid in Kirchner semi-solid medium (in which the drug decayed rapidly), and less frequently with streptomycin and PAS. Colonies which appeared in isoniazid-containing tubes were not always found to consist of drug-resistant organisms on subculture. But when 2-3 days were allowed for microcolonies to appear before the drug was added, then the few large colonies which subsequently developed were found to contain truly resistant organisms. Strains resistant to isoniazid or streptomycin were distinguished from sensitive strains more easily than in liquid media. PAS sensitivity tests gave sharp and consistent end-points in Fisher and Kirchner semi-solid agar even when large inocula were used, though the actual level of the M.I.C. varied greatly with inoculum size, and with different strains. This may be partly accounted for by the reversal of PAS by *p*-aminobenzoic acid (PAB). In Dubos medium readable end-points were often obtained only with small inocula. For all three drugs it seemed that Fisher semi-solid agar gave the most satisfactory results.

The use of semi-solid agar media for the rapid culture of *Mycobacterium tuberculosis* has been previously described (Knox, 1955; Knox, Swait & Woodroffe, 1956). It was mentioned there that such media were found to be particularly useful for the performance of drug sensitivity tests. The present paper gives the results of further experience supporting this, and emphasizes the advantages of this type of medium for assessing the drug susceptibility of *M. tuberculosis*.

METHODS

The methods used were essentially the same as in the previous papers, but will be briefly summarized here. The media mainly used were those of Kirchner, prepared as described by Mackie & McCartney (1953) but without penicillin, of Fisher (1952) and of Dubos (Dubos & Davis, 1946). These media were used with the addition of agar (0.125 %) as semi-solid media, or without it as liquid media.

The organisms used were *Mycobacterium tuberculosis* H37Rv strain, variants of this strain made resistant in the laboratory to isoniazid or streptomycin, and *p*-aminosalicylic acid (PAS) resistant strains of *M. tuberculosis* obtained from Dr W. Steenken, Jun., Trudeau, New York, and from Mr E. M. Bavin, Smith and Nephew Research Ltd., Hunsdon, Hertfordshire. The inoculum was 0.02 ml. of a 10- to 14-day culture in Dubos liquid medium, unless otherwise stated. In some experiments more dilute inocula than this were used, and viable counts were frequently done as well by inoculating serial dilutions in semi-solid Kirchner agar.

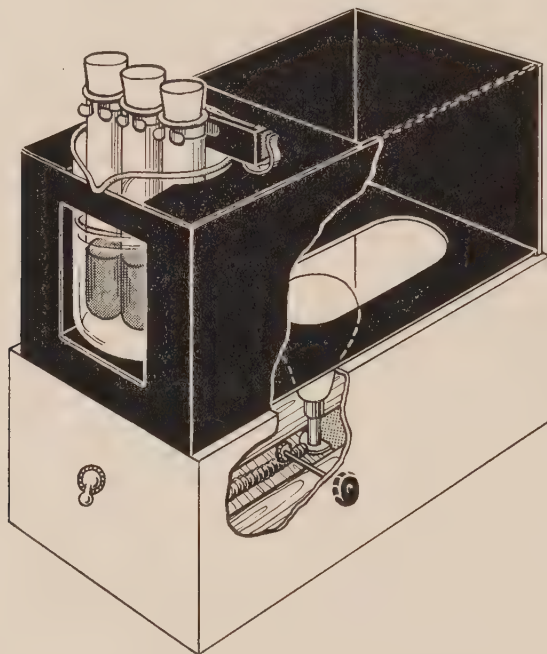


Fig. 1. A diagram of the apparatus used for observing the growth of *Mycobacterium tuberculosis* in semi-solid agar.

Results were recorded by inspection of tubes at frequent intervals. Both observation and recording of results were greatly helped by the improved viewing method described previously (Knox, *et al.* 1956) and shown in Fig. 1, while in many experiments the photographic apparatus described by Engel & Knox (1957) made it easy to obtain permanent serial records.

RESULTS

General appearance of drug sensitivity tests in semi-solid media

The general features of the growth of *Mycobacterium tuberculosis* in semi-solid media have been fully described in the previous papers, and the differences between media with and without Tween 80 have been emphasized. Here, certain general features of drug-sensitivity tests in Kirchner semi-solid agar

medium (without Tween) will first be described. When a large inoculum was used, growth could first be seen in the drug-free control tube in the form of uncountable minute colonies visible with a hand-lens ($\times 6$) under proper illumination as early as 48 hr. after inoculation. This early growth could be easily distinguished from complete inhibition of growth which occurred in tubes containing high drug concentrations and, although the sharpness of the transition between the two extremes varied with the size of inoculum, with the drug used and with the spacing of the drug dilutions, a fairly accurate preliminary reading could be obtained in 2 days. After 3–4 days of incubation growth appeared as a diffuse band easily seen by naked eye and extending for some distance below the surface of the medium, and an end-point giving the 'minimum inhibitory concentration' (M.I.C.), at least for the majority of the organisms inoculated, was sharply defined. On longer incubation growth began to appear in tubes beyond the initial end-point though this itself could still be defined. Depending on the exact conditions used, this delayed growth ranged from innumerable minute colonies to a few large isolated colonies, but it was seldom as profuse, even after long incubation, as growth in the drug-free control tube or in other tubes containing drug concentrations below the initial M.I.C. In general, it may be said that the initial end-point indicated the average drug sensitivity of a bacterial culture (M.I.C.), while the delayed growth of numerous or isolated colonies was partly at least a measure of the variability among different individuals within a bacterial population.

These general features of drug sensitivity tests in semi-solid media are illustrated by several photographs showing the sensitivity to isoniazid of *Mycobacterium tuberculosis* H37Rv as read after different periods of incubation in Kirchner semi-solid agar (Pl. 1). Although, as mentioned above, a sharp end-point was definable in 2 days at a magnification of $\times 6$ and easily readable at 3 days with the naked eye, it was not easy to reproduce these early readings photographically. Photographs taken on the fourth day, however, show the initial end-point clearly. Photographs taken after longer periods of incubation show the nature of the 'shift to the left' which occurred in this medium. The appearance of isolated colonies in increasing drug concentrations on continued incubation is well shown.

Comparison between drug sensitivity tests in semi-solid and liquid media

Table 1 illustrates the contrast between drug sensitivity tests in liquid and in semi-solid media of the same basal composition. In the experiment recorded in Table 1 isoniazid-sensitivity tests were performed with the H37Rv strain and an isoniazid-resistant variant of it in Kirchner liquid and semi-solid agar media. In the liquid medium the M.I.C. of isoniazid for the sensitive strain was hard to determine. Growth in the control tube consisted of coarse granules, which were replaced in tubes containing increasing drug concentrations by finer or less numerous granules, but there was no sharp line of demarcation. In the semi-solid medium, on the other hand, a sharp end-point was readable at 2–3 days and, as shown in Pl. 1, could be photographed at 4 days, while the subsequent 'shift to the left' could be expressed

quantitatively, consisting as it did of decreasing numbers of colonies as the drug concentration was increased. Differences between the sensitive and resistant strain were also shown more clearly in the semi-solid medium since they too could be expressed quantitatively. In the liquid medium both sensitive and resistant strains eventually grew in 10 μ g. isoniazid/ml. and were distinguished mainly by the time taken for growth to occur in that concentration

Table 1. *Isoniazid sensitivity tests in liquid and semi-solid media*

In this experiment doubling dilutions were used, but for brevity only fourfold dilutions are reported.

Strain of <i>M. tuberculosis</i>	Medium	Period incubated at 37° (days)	Concentration of isoniazid (μ g./ml.)						
			10.0	2.5	0.625	0.156	0.039	0.009	0
H37 Rv	Liquid	4	Tr	Tr	Tr	Tr	Tr	±	+
		11	±	±	±	±	+	+ ±	+ ±
		30	+	++	++	++	+++	+++	+++
	Semi-solid	4	0	0	0	0	0	SC	SC
		11	0	0	0	0	Unc	SC	SC
		30	1 colony	Unc	SC	C	C	C	C
I.N.H.-resis- tant variant (R1)	Liquid	4	±	±	±	±	±	±	±
		11	+	+	+	+	+	+	+
		30	++	++	++	++	++	++	++
	Semi-solid	4	Unc	Unc	Unc	Unc	Unc	Unc	Unc
		11	SC	SC	SC	SC	SC	SC	SC
		30	C	C	C	C	C	C	C

In liquid medium, + signs indicate approximate amount of granular growth, Tr=trace of growth.

In semi-solid medium, C=confluent, SC=semi-confluent growth, Unc=uncountable colonies.

of the drug. In the semi-solid medium, however, the two strains were easily distinguished since the growth of the resistant strain in that concentration was rapid and equal to its growth in the control tube, whereas growth of the sensitive strain occurred only after a long delay and when it did occur, consisted of only one colony.

Sensitivity patterns with different drugs

Isoniazid. One of the characteristic features of isoniazid-sensitivity tests in most media is the 'shift to the left' which makes the reading of an end-point in liquid media difficult (Knox, King & Woodroffe, 1952). As shown above, semi-solid media combine the advantages of a sharp early end-point with a clear picture of the development of presumptively resistant colonies on continued incubation (see Pl. 1). It was found, however, that there were considerable differences between different media. In all the three media here used (Kirchner, Fisher, Dubos) the initial end-point for the H37 Rv strain was 0.05–0.01 μ g. isoniazid/ml., when read on the first possible occasion (2–4 days), but a shift to the left occurred rapidly in Kirchner medium, rather more slowly in Dubos medium and more slowly still in Fisher medium. A typical experiment is illustrated in Table 2.

In several experiments isoniazid-sensitivity tests were carried out with inocula of different sizes. The end-point was somewhat higher with very heavy

Table 2. *Isoniazid sensitivity of Mycobacterium tuberculosis H37 Rv strain in three semi-solid media*

In this experiment doubling dilutions of drug were used but for brevity not all of these have been included.

Medium	Period incubated at 37° (days)	Concentration of isoniazid ($\mu\text{g./ml.}$)						
		2.5	0.625	0.156	0.039	0.019	0.009	0
Kirchner	3	0	0	0	0	0	SC	SC
	7	0	0	0	0	0	SC	SC
	17	0	0	0	Unc	Unc	C	C
	31	Unc	Unc	SC	C	C	C	C
Dubos	3	0	0	0	0	0	SC	SC
	7	0	0	0	0	SC	SC	SC
	17	0	0	Unc	SC	C	C	C
	31	0	Unc	SC	C	C	C	C
Fisher	3	0	0	0	0	0	SC	SC
	7	0	0	0	0	0	SC	SC
	17	0	0	0	SC	C	C	C
	31	0	0	50 colonies	C	C	C	C

C = confluent, SC = semi-confluent growth, Unc = uncountable colonies.

than with dilute inocula, but the extent of this effect varied a good deal with the period of incubation. When readings were made at the earliest possible moment, end-points were around 0.01–0.05 $\mu\text{g./ml.}$ for a wide range of inocula. But after incubation, even for only 10 days, differences became obvious, and the shift to the left was very rapid with large but negligible with small inocula.

It has already been shown (Table 1) that in spite of this shift to the left, drug-sensitive and drug-resistant strains were more easily distinguished in semi-solid than in the corresponding liquid media. The isoniazid-resistant variant of the H37Rv strain grew well and rapidly in 10 $\mu\text{g.}$ isoniazid/ml., but not, usually, much above 20 $\mu\text{g./ml.}$ When more closely spaced drug dilutions were used, however, it was seen that a slight 'shift' occurred, although relatively much less than with the sensitive strain. This is illustrated in Table 3.

Table 3. *Isoniazid sensitivity of drug-resistant variant of Mycobacterium tuberculosis H37 Rv strain*

Period incubated at 37° (days)	Concentration of isoniazid ($\mu\text{g./ml.}$)						
	28	26	24	22	20	10	0
3	0	0	0	Unc	SC	C	C
21	37 colonies	Unc	C	C	C	C	C

C = confluent growth, SC = semi-confluent growth, Unc = uncountable colonies.

Streptomycin. Table 4 shows the streptomycin sensitivity of the H37Rv strain in Kirchner, Fisher and Dubos semi-solid media; the initial end-point at 3 days was about 0.15 $\mu\text{g./ml.}$ in all 3 media. On continued incubation the

end-point shifted slightly in Fisher and in Dubos media, but more extensively in Kirchner medium. But in none of the media was the shift as great as in the corresponding media containing isoniazid. The initial end-point varied with the size of inoculum more conspicuously with streptomycin than with isoniazid. On the other hand, the subsequent shift to the left was much less conspicuous with streptomycin, even when heavy inocula were used, than it was with isoniazid with much smaller inocula. Streptomycin-resistant strains grew well in semi-solid agar and could easily be distinguished from sensitive strains.

Table 4. *Streptomycin sensitivity of Mycobacterium tuberculosis H37 Rv strain in three different semi-solid media*

Medium	Period incubated at 37° (days)	Concentration of isoniazid (µg./ml.)					
		2.5	0.625	0.156	0.039	0.009	0
Kirchner	3	0	0	0	SC	SC	SC
	7	0	Unc	SC	SC	SC	SC
	17	0	C	C	C	C	C
	31	50	C	C	C	C	C
Dubos	3	0	0	0	SC	SC	SC
	7	0	0	0	SC	SC	SC
	17	0	0	0	SC	SC	SC
	31	0	0	Unc	C	C	C
Fisher	3	0	0	0	SC	SC	SC
	7	0	0	SC	SC	SC	SC
	17	0	0	SC	C	C	C
	31	0	4	C	C	C	C

C=confluent growth, SC=semi-confluent growth, Unc=uncountable colonies. Figures give number of colonies when these were countable.

p-Aminosalicylic acid (PAS). The sensitivity of the H37 Rv strain to PAS was about the same in all the three semi-solid media (though the M.I.C. was usually slightly less in Kirchner and Fisher than in Dubos semi-solid agar). When cultures were examined daily after inoculation large numbers of minute colonies (visible only with a lens $\times 6$) could be seen in the first 2 or 3 days in tubes containing high PAS concentrations. In these tubes, however, this 'growth' did not progress, and by the fifth or sixth day it was usually possible to establish a M.I.C. which shifted very little on further incubation. The exact position of this end-point varied considerably with the size of inoculum and the medium, ranging from 0.5 µg./ml. with an undiluted inoculum to 0.05 µg./ml. with a 10^{-4} dilution. The end-point was usually much sharper in Kirchner and Fisher semi-solid media than it was either in the corresponding liquid media or in Dubos medium, either liquid or semi-solid, in which when large inocula were used it was often impossible to define an end-point at all. Table 5 shows the effect of inoculum size on the PAS sensitivity of the H37 Rv strain in liquid and semi-solid Kirchner agar read at 8 and 29 days. Table 6 shows the PAS sensitivity of 4 strains of *Mycobacterium tuberculosis* in Kirchner semi-solid agar. When readings were taken at 15 days, the H37 Rv strain was

found to be sensitive to 0.45 μg . PAS/ml. or less, whether the inoculum was undiluted or diluted 1 in 100. With one resistant strain (R. 10) growth occurred in 125 μg . PAS/ml. from both inocula, but the M.I.C. for two other strains was found to be highly dependent on inoculum size, decreasing with strain R. 14 from about 125 to 8 μg ./ml., and with strain R. 15 from 31 to 2 μg ./ml. when undiluted inocula were compared with 1 in 100 dilutions.

Table 5. *Sensitivity of Mycobacterium tuberculosis H37 Rv strain of p-aminosalicylic acid (PAS) in liquid and semi-solid media*

Medium	Inoculum	Period incubated at 37° (days)	Concentration of PAS (μg ./ml.)							
			10.0	1.0	0.5	0.1	0.05	0.01	0.001	0
Liquid Kirchner	Undiluted	8	0	0	±	±	+	+	+	+
		29	±	±	±	+	++	++	++	++
	10 ⁻²	8	0	0	0	0	0	±	±	±
		29	0	0	0	±	+	+	++	++
	10 ⁻⁴	8	0	0	0	0	0	0	0	0
		29	0	0	0	Tr	Tr	±	±	±
Semi-solid Kirchner agar	Undiluted	8	0	0	0	SC	C	C	C	C
		29	0	0	0	C	C	C	C	C
	10 ⁻²	8	0	0	0	0	0	Unc	SC	SC
		29	0	0	0	SC	C	C	C	C
	10 ⁻⁴	8	0	0	0	0	0	0	0	0
		29	0	0	0	22	29	50	50	50

C=confluent growth, SC=semi-confluent growth, Unc=uncountable colonies. Figures give number of colonies when these were countable.

Table 6. *Sensitivity of 4 strains of Mycobacterium tuberculosis to p-aminosalicylic acid (PAS) in Kirchner semi-solid agar*

Inoculum		Concentration of PAS (μg ./ml.)					
		1.25	31.2	7.8	1.95	0.45	0
H37 Rv	Undiluted	0	0	0	0	0	SC
	1/100	0	0	0	0	0	SC
R. 15	Undiluted	0	0	Unc	SC	SC	SC
	1/100	0	0	0	0	Unc	Unc
R. 14	Undiluted	0	SC	SC	SC	SC	SC
	1/100	0	0	0	Unc	Unc	Unc
R. 10	Undiluted	SC	SC	SC	SC	SC	SC
	1/100	SC	SC	SC	SC	SC	SC

Readings after 15 days of incubation. Doubling dilutions of PAS were used but for brevity only fourfold dilutions are shown. C=confluent growth, SC=semi-confluent growth, Unc=uncountable colonies. 0=no growth.

It was shown by Youmans, Raleigh & Youmans (1947) that the action of PAS on *Mycobacterium tuberculosis* could be reversed by *p*-aminobenzoic acid (PAB), and the variability in PAS sensitivity tests may well be related to differences in the concentration of PAB in different media or in its production by different strains. Table 7 shows that when PAB was incorporated in

semi-solid Kirchner agar it reversed the action of PAS in what appears to be a competitive way. But it was not necessary to incorporate the PAB into the medium. It could be added (in a strength calculated to give a required concentration by diffusion through the medium) to tubes which had already been inoculated and incubated up to several days. Table 8 shows that even when added to PAS-containing tubes after incubation for 5 days PAB showed some annulling action, though this was much slower than after shorter periods of incubation.

Table 7. *Effect of p-aminobenzoic acid (PAB) in annulling effect of p-aminosalicylic acid (PAS)*

Medium: Kirchner semi-solid. Readings after 6 days of incubation at 37°.

Concentration of PAB (μg./ml.)	Concentration of PAS (μg./ml.)									
	10.0	5.0	1.0	0.5	0.1	0.05	0.01	0.005	0.001	0
0	0	0	0	0	SC	SC	SC	SC	SC	SC
					small	small	small			
1.0	0	0	SC	SC	SC	SC	SC	SC	SC	SC
5.0	0	SC	SC	SC	SC	SC	SC	SC	SC	SC
10.0	SC	SC	SC	SC	SC	SC	SC	SC	SC	SC

SC=semi-confluent growth.

Table 8. *Annulment of effect of p-aminosalicylic acid (PAS) by p-aminobenzoic acid (PAB) added at intervals after inoculation*

Organism: *Mycobacterium tuberculosis*, strain H37 Rv. Medium: Kirchner semi-solid.

Period of incubation (days) before adding PAB (10 μg./ml.)	Period incubated at 37° (days)	PAS concentration (μg./ml.)										
		8.0	4.0	2.0	1.0	0.5	0.25	0.125	0.062	0.031	0.016	0
0	7	C	C	C	C	C	C	C	C	C	C	C
	12	C	C	C	C	C	C	C	C	C	C	C
	19	C	C	C	C	C	C	C	C	C	C	C
1	7	0	C	C	C	C	C	C	C	C	C	C
	12	C	C	C	C	C	C	C	C	C	C	C
	19	C	C	C	C	C	C	C	C	C	C	C
2	7	0	0	0	0	C	C	C	C	C	C	C
	12	C	C	C	C	C	C	C	C	C	C	C
	19	C	C	C	C	C	C	C	C	C	C	C
4	7	0	0	0	0	0	0	C	C	C	C	C
	12	0	0	0	C	C	C	C	C	C	C	C
	19	SC	C	C	C	C	C	C	C	C	C	C
5	7	0	0	0	0	0	0	C	C	C	C	C
	12	0	0	0	0	C	C	C	C	C	C	C
	19	0	C	C	C	C	C	C	C	C	C	C
No PAB added	7	0	0	0	0	0	0	C	C	C	C	C
	12	0	0	0	0	0	0	C	C	C	C	C
	19	0	0	0	0	0	SC	C	C	C	C	C

0=no growth, SC=semi-confluent, C=confluent growth.

Summary of the differences between different drugs

The results here described show that the drug-sensitivity patterns for isoniazid, streptomycin and PAS in semi-solid agar showed characteristic differences. With isoniazid, the initial end-point (M.I.C.) was about 0.01–0.05 $\mu\text{g./ml.}$ and was often little affected either by inoculum size or the formula of the medium. But on continued incubation 'resistant' colonies developed far more rapidly in Kirchner than in Dubos or Fisher semi-solid agar, and their number was roughly proportional to the size of the inoculum. With streptomycin, on the other hand, it was the initial end-point which was affected both by the size of the inoculum and by the nature of the medium. The M.I.C. for streptomycin was four- to eightfold higher in Kirchner than in Dubos or Fisher semi-solid agar in which it was 0.15–0.3 $\mu\text{g./ml.}$ With streptomycin the development of 'resistant' colonies did not spread over so wide a range of concentrations as with isoniazid. However, as will be seen later, it was easier to select variants permanently resistant to streptomycin than to isoniazid. With PAS the initial end-point was not always as clearly defined in the first 3–4 days of incubation as it was with the other two drugs, but usually sharp end-points were obtained at 7 days, especially in Kirchner and Fisher semi-solid agar, even when large inocula were used. Although the M.I.C. varied greatly with inoculum size it showed little change on further incubation and the development of 'resistant' colonies was much slower than with either of the other two drugs. In several experiments it was found that, with isoniazid or streptomycin, when growth occurred in increasing concentrations of drug it consisted of progressively smaller numbers of colonies, but the colonies which did develop eventually reached normal size. With PAS, on the other hand, the size of the colonies decreased steadily with increasing drug concentration, but the numbers were much less affected. This interesting effect was not consistently observed and requires further investigation.

Inactivation of isoniazid, streptomycin and p-aminosalicylic acid in uninoculated media

It was natural to suspect that differences in initial end-point and in its stability might be related to the chemical stability of the different drugs in different media. Solutions containing known initial concentrations (4–5 $\mu\text{g./ml.}$) of isoniazid, streptomycin or PAS were incubated at 37°; samples were removed at intervals and the drug concentration determined by the serial dilution method using for each assay the end-point given by the H37 Rv strain in Dubos liquid medium after 7 days of incubation. The results are shown in Fig. 2. None of the drugs decayed significantly in distilled water at 37° over a period of 4 weeks. Streptomycin decayed slowly in all 3 media. Isoniazid decayed very rapidly in Kirchner and in Dubos medium but more slowly in Fisher medium. PAS showed no significant decay in any of the media (Fig. 2). The presence of PAS did not affect the decay of isoniazid, nor did isoniazid affect the stability of PAS. In one experiment the decay of isoniazid in semi-solid Kirchner medium was followed directly by inoculating

the H37Rv strain on to the surface of drug-containing tubes incubated for different times at 37°. The decay was of the same order as was found in liquid Kirchner medium.

The drug sensitivity of organisms growing in drug-containing semi-solid agar

On many occasions attempts were made to measure the drug sensitivity of organisms growing in semi-solid media containing various concentrations of isoniazid or streptomycin. Several methods were used. Agar at the concentration of 0.125% used, while solid enough to give discrete colonies when

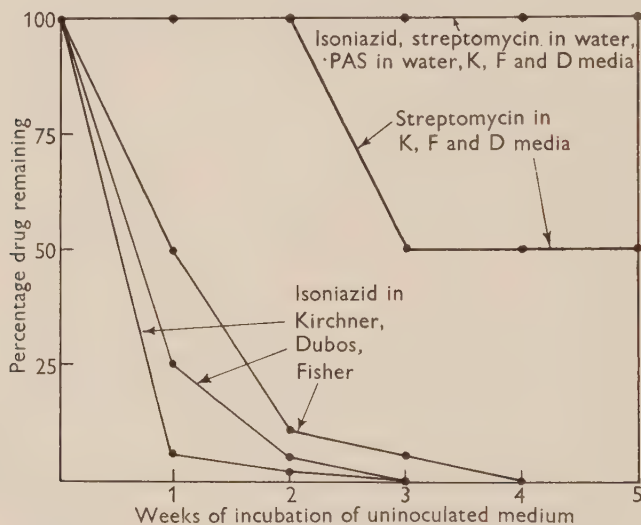


Fig. 2. Decay of drugs in uninoculated media. K=Kirchner; F=Fisher; D=Dubos.

suitably inoculated, was also liquid enough to be sucked up in a Pasteur or dropping pipette. A single colony could thus be picked up and transferred to another tube and then shaken up with Dubos or other suitable liquid medium. When separate colonies were not available, then a confluent or semi-confluent growth could be similarly transferred in a pipette. Inoculated tubes were then incubated and after 7–14 days the drug sensitivity of the culture was determined in semi-solid Dubos agar. It was found that colonies growing in high drug concentrations were not necessarily as resistant as would be suggested by the drug concentration in which they originally grew, though many of them did show increased drug resistance when tested in this way. It was interesting to find that although presumptively 'resistant' colonies occurred with greater frequency with isoniazid than with streptomycin, drug resistance in the sub-cultures was more often found with streptomycin than with isoniazid. It is possible of course that any truly resistant variants, which might have grown in drug-containing media, might have been largely eliminated by the process

of subcultivation in drug-free medium involved in the method of sensitivity testing here used. Preliminary experiments have shown that single colonies can be transferred from semi-solid agar medium and, after shaking in Dubos or other suitable medium, can be used without subculture in drug-free medium for sensitivity tests. Similarly a 4-day culture of the H37Rv strain in Dubos semi-solid agar could be used for direct inoculation by a Pasteur pipette with a set of tubes containing serial drug dilutions in semi-solid agar and the drug sensitivity estimated in 2 days with a hand-lens and in 3–4 days by naked eye. This technique is being developed to investigate afresh this problem of the true drug sensitivity of presumptively resistant colonies growing in drug-containing media.

Selection of drug-resistant variants in semi-solid agar media

The early development of microcolonies of *Mycobacterium tuberculosis* in semi-solid agar offered an opportunity to study the effect of drugs added to known numbers of organisms at different stages in their development. Only a few preliminary experiments have been done, but some interesting results have been obtained by adding isoniazid, streptomycin or PAS to 3-day cultures of the H37Rv strain in semi-solid agar. It was mentioned earlier that with a heavy inoculum growth could be seen at this stage to consist of innumerable minute colonies. Drugs added to such cultures diffused rapidly through the medium and arrested or delayed further growth. With PAS the colonies continued for 2–3 days to increase slowly in size as compared with colonies in drug-free medium, but usually no large colonies developed. With isoniazid or streptomycin, on the other hand, the growth of nearly all colonies was completely arrested, but a few colonies continued to grow at about the normal rate. These colonies, when their drug sensitivity was determined by the method described above, were found to be much more resistant than similar colonies growing in media which had contained drugs from the time of inoculation. This would suggest that a more accurate picture of the mutation rate to drug resistance might be obtained from adding drugs to young cultures in a medium in which they are actively multiplying rather than to older cultures which are exposed to new medium and drug at the same time.

DISCUSSION

The differences obtained with the three media here used show clearly some of the difficulties in the interpretation of drug sensitivity tests on cultures of *Mycobacterium tuberculosis*. Both the initial end-point and the number of presumptively resistant organisms are greatly influenced by the medium used, and it is not possible at present to say which medium gives the more accurate picture of the average sensitivity of a culture and of the distribution of resistant individuals in it. These difficulties, however, are inherent in any of the tests at present used and can be to some extent overcome by using a strain such as the H37Rv strain of *M. tuberculosis* as a standard for comparison.

The method of serial drug dilutions in liquid media frequently used for

drug-sensitivity tests gives at best a fairly accurate picture of the average drug sensitivity of the majority of the organisms in a bacterial culture. If a culture is fairly homogeneous in this respect, then the method gives a sharp end-point between 'no growth' and 'growth equal to the drug-free control', even when closely spaced (e.g. doubling) dilutions are used. But when there is a good deal of variation within the bacterial population the method loses much of its value. A sharp end-point can then be obtained only by increasing the spacing of the dilutions (say to tenfold) with a great loss of accuracy. If doubling or fourfold dilutions are still used, then a sharp end-point is no longer obtained. These difficulties are especially troublesome with sensitivity tests for tubercle bacilli in liquid media. Provided a small inoculum is used and conditions are carefully standardized it may be possible to obtain a fairly sharp end-point if sensitivity tests are read at the earliest possible moment. But all strains do not grow at the same speed and when on continued incubation a shift in the end-point occurs, it is impossible to tell with liquid media whether this is due to delayed growth of most of the organisms in the culture or only of a few individuals different from the average. Further, no information is given about the frequency of drug-resistant mutants in a bacterial population.

Fully solid media, on the other hand, which do give this information have many disadvantages which have already been discussed (Knox *et al.* 1956). Growth is usually rather slow, the risks of contamination or dehydration of the medium are difficult to avoid and cultures or sensitivity tests cannot conveniently be repeatedly inspected and assessed at a glance. Semi-solid media, however, combine the advantages of liquid and solid media without the disadvantages of either. The results shown in this paper suggest that though one medium might be the best with one drug but not so good with another, the medium most suitable for all three drugs was Fisher semi-solid agar.

Since both viable counts and drug-sensitivity tests can be so easily and conveniently performed, semi-solid media open up new possibilities for the intensive study of drugs and combinations of drugs, both for bacteriostatic and bactericidal activity. Since many substances added to low agar concentrations diffuse rapidly through the media, they can also be used to investigate the action of reversing agents added at different periods after inoculation, for example, the effect of *p*-aminobenzoic acid in reversing the action of *p*-aminosalicylic acid or of haemin in reversing the action of isoniazid. In the examination of clinical material too, semi-solid media have shown great promise. Rapid results have been obtained both with 'indirect' tests on cultures isolated from patients' sputa and also with direct tests on sputum concentrates. These results are reported in a separate communication (Knox & Skinner, 1957).

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EXPLANATION OF PLATE

Isoniazid sensitivity of H37Rv strain of *M. tuberculosis* recorded after different times of incubation. The tubes contained the following isoniazid concentrations ($\mu\text{g./ml.}$): 7, 0.6; 6, 0.3; 5, 0.16; 4, 0.08; 3, 0.04; 2, 0.02; 1, 0.01; C (control) 0. Note that in the last two photographs tubes 2 and 1 have been omitted, so that tube 3 is now next to the control tube.

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Acriflavine-resistant Mutants of *Aspergillus nidulans*

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SUMMARY: In three independently obtained mutant strains of *Aspergillus nidulans*, resistant in different degrees to acriflavine, resistance is due, in each case, to mutation in a single gene. Two of the mutant alleles, *ACR1* and *ACR3*, are semi-dominant and either allele confers a high degree of resistance. These alleles are located about 23 units distal to the *w* (white conidia) locus and are presumably allelic. A cross involving these two alleles in repulsion gave 0.1 % sensitives. A third mutant allele (*acr2*) is also located on the *w* chromosome, but on the other arm about 25 units distal to the *ad1* locus and over 100 units distant from the *ACR1* and *ACR3* loci. This allele, which confers relatively slight resistance, is almost completely recessive. Diploid strains which carry any allele for resistance in heterozygous condition give, by vegetative segregation, haploid and homozygous diploid resistant types which are preferentially selected on medium with acriflavine. The use of this technique for the automatic selection of vegetative segregants provides an additional tool for analyses through the parasexual cycle.

Various mutant types have been used in genetic investigations with *Aspergillus nidulans*. Such types include those differing from wild type in conidial colour, colony morphology, nutritional requirements and ability to secrete certain enzymes (Pontecorvo, 1953). The present paper reports the extension of the range of mutant types to include those able to grow in the presence of certain substances which are inhibitory to the growth of the wild type. The aim in obtaining such resistant strains was that they should be used not only as a study in themselves, but also to provide an extra tool in the selection of vegetative segregants from heterozygous diploid strains. Strains of filamentous fungi carrying diploid nuclei give, though with a low frequency, mitotic segregants which result either from mitotic crossing-over (Pontecorvo & Roper, 1952, 1953) or from haploidization (Pontecorvo & Roper, 1953; Pontecorvo, Tarr Gloor & Forbes, 1954). Genetic analyses based on the use of this parasexual process have been carried out in *A. nidulans* (Pontecorvo & Roper, 1953; Pontecorvo *et al.* 1954; Pontecorvo & Käfer, 1956), in *A. niger* (Pontecorvo, 1952; Pontecorvo, Roper & Forbes, 1953) and in *Penicillium chrysogenum* (Pontecorvo & Sermonti, 1954). The isolation and identification of segregants have been made in several ways: (i) visually, by isolating conidial heads of a colour different from those of the heterozygote (Pontecorvo & Roper, 1953); (ii) nutritionally, by isolating segregants requiring some nutrient not required by the heterozygote (Forbes, 1952); (iii) nutritionally by the selection, through the use of suppressors, of segregants independent of some nutrient which the starting strain required (Pontecorvo & Käfer, 1956);

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(iv) nutritionally by the selection of prototrophs from diploids carrying alleles in repulsion (Roper & Pritchard, 1955). Only the last two of these techniques are automatic. It was hoped that either recessive or semi-dominant alleles conferring resistance would provide a further automatic technique of selection.

Aspergillus nidulans is surprisingly resistant to a wide range of substances which might be expected to be growth-inhibitory or toxic. Copper sulphate, organo-arsenical compounds and a wide range of dyestuffs even at high concentrations do not inhibit growth sufficiently to allow the selection of clear-cut resistant strains. Of many substances tested acridine was the first to give, when used at workable concentration, effective inhibition of growth.

METHODS

The general techniques used throughout the present work were the same as those described by Pontecorvo (1953).

Media. Minimal medium (MM) Czapek-Dox with 2% glucose. Complete medium (CM), a complex medium containing yeast extract, casein hydrolysate, hydrolysed nucleic acid, vitamins, etc. Incubation was at 37°.

Organisms. Sensitive starting strains were selected from the laboratory stock of mutant strains. The symbols used to designate mutant alleles are those used previously (Pontecorvo, 1953). The mutant alleles used in this work, and the phenotypes these alleles determine, are listed in Table 1.

Table 1. *Phenotypes determined by mutant alleles of Aspergillus nidulans; and their symbols*

Symbol	Phenotype
<i>y</i>	Yellow conidia (wild type conidia are green)
<i>w</i>	White conidia
<i>ad</i>	Adenine requirement
<i>pyro</i>	Pyridoxin requirement
<i>sd</i>	Sulphite requirement
<i>bi</i>	Biotin requirement
<i>paba</i>	<i>p</i> -Aminobenzoic acid requirement

Isolation of mutants. Ten strains, carrying various mutant alleles, were selected from the laboratory stock and tested for sensitivity to acridine. Petri plates of CM with various concentrations of acridine were spread with conidial suspensions so that each plate had about 10⁷ conidia; these were examined after 3 days of incubation. All the ten strains were sensitive to acridine to approximately the same degree.

Mutants were selected by plating conidia of sensitive strains on CM containing 0.005% (w/v) acridine. This concentration of acridine is approximately three times that required to prevent germination of sensitive conidia plated at a density of 10⁷ conidia/dish. In fact, at such high densities of conidia some diffuse growth was obtained round the edges of the dishes where masses of conidia had clumped. That this growth was due to adaptation and not mutation was shown by the transfer of conidia from such growth to further acridine medium on which it invariably failed to grow. The vigorously

growing mutant types could be clearly distinguished from growth due to adaptation.

The first mutant was obtained from the plating of the strain *paba1 y*; the second from *ad15 paba1 y* and the third from *ad1 pyro4 sd bi1*. Although several resistant colonies were obtained at each plating only one was selected from each so as to avoid investigating a clone. Different starting strains in each case eliminated possible difficulties due to contamination. No attempt was made to estimate accurately the frequency of acriflavine-resistant mutants, but the yield of resistant colonies in each selection varied between 1 in 2×10^7 and 1 in 10^8 .

Detailed genetical analysis of the three selected resistant strains is described below. It was shown that in each strain a single mutant allele determined resistance. The three alleles were designated *ACR1*, *acr2* and *ACR3*.

Degree of sensitivity of wild type and mutants

Haploids. Sensitivity to acriflavine is not significantly affected by the concentration of vitamins or amino acids, and is independent of pH value within the range pH 4–7. However, sensitivity is dependent on the concentration of nucleic acid (cf. McIlwain, 1941). When the concentration of hydrolysed nucleic acid in the CM was increased 3- to 20-fold, resistance to acriflavine of both sensitives and resistants was increased about 3- to 8-fold. For this reason quantitative tests of sensitivity were made with standardized batches of CM.

Attempts to measure resistance by means of linear growth rate on different acriflavine concentrations are liable to considerable error owing to the relatively slow growth rate of this organism. Further, when this method was applied to heterozygotes, sectors, due to the selection of segregants (see below), made accurate measurement impossible.

To determine the resistance of a strain quantitatively, each of a series of plates of CM, with or without acriflavine, was spread with about 80 conidia. Ten replicates were used in each series so that for any one concentration of acriflavine 800 conidia of a strain were tested for ability to produce a colony after 3 days of incubation. Colony counts so obtained were expressed as a percentage of the counts on CM without acriflavine. Figure 1 shows the curves obtained for strains each carrying one of the mutant alleles *ACR1*, *acr2* or *ACR3*; wild type is shown for comparison. The degree of resistance conferred by each allele is different, though for general working purposes *ACR1* and *ACR3* are sufficiently similar to be interchangeable. That the differences in resistance were determined by the mutant alleles themselves and not by modifiers was shown by outcrossing and testing recombinants with each allele. Such recombinants always showed a degree of resistance not significantly different from that of the parent strain.

The three resistant strains also showed a greater resistance to crystal violet and malachite green than did wild type. The results with these two inhibitors were not always perfectly reproducible and resistance to them was not further investigated.

Diploids. Using the technique of Roper (1952) 8 strains carrying diploid nuclei of the following genotypes with respect to acriflavine resistance were prepared: $ACR1/ACR1$, $acr2/acr2$, $ACR3/ACR3$, $ACR1/+$, $acr2/+$, $ACR3/+$, $ACR1/ACR3$ and wild type. All the strains were also heterozygous for other markers, which determined nutritional requirements and conidial colour.

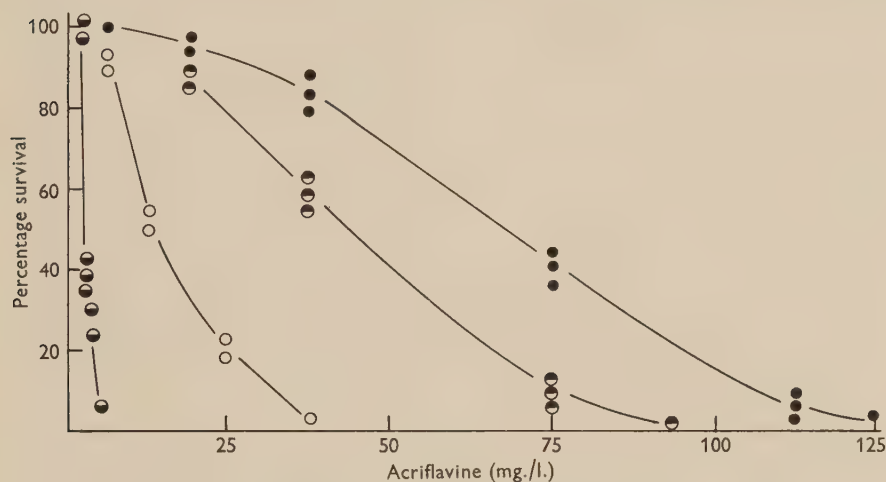


Fig. 1. Survival of conidia on CM with acriflavine.

●, $ACR1$; ◐, $ACR3$; ○, $acr2$; ◑, wild type.

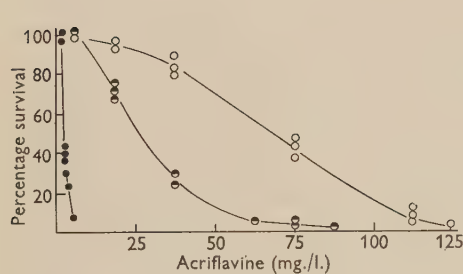


Fig. 2. Survival of conidia on CM with acriflavine. ○, $ACR1$; ◐, $ACR1/+$; ●, wild type.

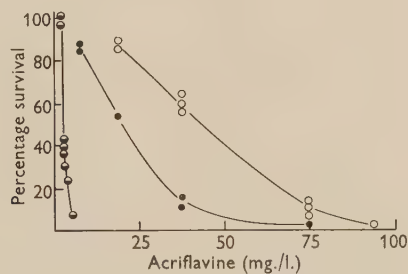


Fig. 3. Survival of conidia on CM with acriflavine. ○, $ACR3$; ◐, $ACR3/+$; ●, wild type.

Each of the four strains $ACR1/ACR1$, $acr2/acr2$, $ACR3/ACR3$ and the diploid wild type showed a resistance like that of the corresponding haploid. The resistance of the strains $ACR1/+$, $acr2/+$, $ACR3/+$ and $ACR1/ACR3$ are shown in Figs. 2–5. Figs. 2 and 3 show that the alleles $ACR1$ and $ACR3$ are semi-dominant; curves for the heterozygotes lie between those of wild type and the resistant homozygous diploid or the resistant haploid. At anything other than the lowest acriflavine concentrations the genotypes+ (haploid or diploid), $ACR1/+$ and $ACR1$ (or $ACR1/ACR1$) can be distinguished not only in terms of viability but also by colony size (Pl. 1, fig. 1);

the same applies to allele *ACR3*. Not only that, but heterozygotes invariably sector (Pl. 1, fig. 2) on acriflavine medium to give sectors growing at the rate of the haploid or homozygous diploid resistant. Fig. 4 shows the behaviour of the strain *ACR1/ACR3* whose resistance lies between that of its components.

On the other hand, the strain *acr2/+* has very low resistance (Fig. 5) and can only be distinguished from wild type by careful tests of survival or the ability to produce resistant segregants. The allele *acr2* is almost completely recessive.

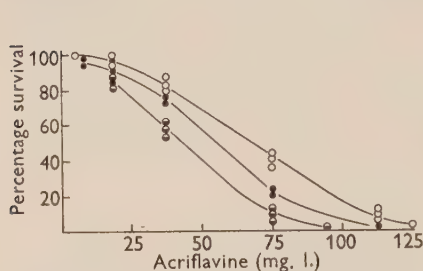


Fig. 4. Survival of conidia on CM with acriflavine. ○, *ACR1*; ●, *ACR3*; ●, *ACR1/ACR3*.

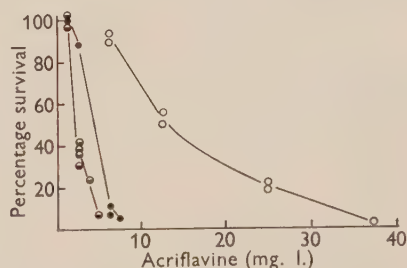


Fig. 5. Survival of conidia on CM with acriflavine. ○, *acr2*; ●, *acr2/+*; ●, wild type.

Heterokaryons. A number of balanced heterokaryons were prepared which were comparable to certain of the above heterozygotes. So far this has been done only for the allele *ACR1*. With respect to this allele the three investigated heterokaryons had nuclei of types: + with +; + with *ACR1*; *ACR1* with *ACR1*. The resistance of the heterokaryons could not be tested on the same basis as the resistance tests discussed above. Growth-rate measurements were made on gradient plates of MM (Szybalski, 1952) in which the lower layer contained acriflavine and the upper did not. Heterokaryons were subcultured by transfer to the gradient plates of hyphal tips from heterokaryotic colonies on minimal medium. Subculture was made to the point on the Szybalski layer plate where the acriflavine concentration was lowest. Such tests showed that the relative degrees of resistance of these three heterokaryons were comparable with the relative degrees of resistance of the corresponding diploids; that is, the allele *ACR1* is semi-dominant in a heterokaryon.

Genetical analysis of the mutants

Meiotic analysis. The determination of the genetical control of resistance and the location of the alleles involved was first made through the sexual cycle; routine techniques being used (Pontecorvo, 1953). From crosses of a sensitive strain to each resistant strain it was shown that mutation of a single gene determined resistance in each strain. The mutant alleles were designated *ACR1*, *acr2* and *ACR3*. The allele *ACR1* was located 23.0 ± 1.9 units from the *w* locus. Subsequent mitotic analysis (see below) showed that this locus was distal to the *w* locus. Similarly the allele *ACR3* was shown to be located in the same region. The allele *acr2* was located on the same chromosome

26.0 ± 2.9 units distal to the *ad1* locus; that is, more than 100 units from the *ACR1* and *ACR3* loci and on the other arm of that chromosome.

Physiological allelism of *ACR1* and *ACR3* could not be tested by the usual practice of observing the phenotype of the double heterozygote in *trans* arrangement since these mutant alleles are semi-dominant.

A cross was made involving the markers: + *ACR1* × *w ACR3*. Ascospores from the cross were plated on CM and colonies tested for resistance or sensitivity. Of 3468 colonies tested 4 (1 white conidia and 3 coloured conidia) were sensitive. Owing to the lack of closely linked markers it is impossible to say whether these sensitives resulted from recombination or reversion. In any case *ACR1* and *ACR3* are very closely linked if not, in fact, allelic.

Mitotic analysis with ACR1 and ACR3. When conidia of the heterozygote *ACR1*/+ or *ACR3*/+ were plated on media with appropriate concentrations of acriflavine the resulting colonies showed sectors after 2–4 days of incubation. Optimal conditions for selection of sectors was given by CM containing c. 0.0025 % (w/v) acriflavine. Such sectors (Pl. 1, fig. 2) grew at the same rate as the haploid or homozygous diploid resistant types and were therefore presumed to be vegetative segregants of genotype *ACR* or *ACR/ACR*. Segregants of such genotype should be able to outgrow the heterozygote on acriflavine medium. Isolates made from the tips of such sectors almost always gave pure cultures which could be immediately tested. Analysis of segregant sectors showed, as expected, that both haploid and diploid segregants arose (Pontecorvo & Käfer, 1956). The data of these authors showed

that from the heterozygotes $\frac{ACR\ w}{+ +} \bullet$ and $\frac{ACR +}{+ w} \bullet$ (\bullet = centromere) haploid segregants were white and coloured respectively.

From the former heterozygote the diploid segregants were, as expected, all *ACR/ACR*; some were *w/w* and others *w/+*, showing that the order of the loci with respect to their centromere is as indicated.

Mitotic analysis with recessive allele acr2. Two techniques for segregant selection are possible with the recessive allele *acr2*. Some conidia formed by a strain *acr2*/+ will have segregant nuclei of type *acr2* or *acr2/acr2*. These can be selected by plating large numbers of conidia formed by the heterozygote on CM containing 0.0013 % (w/v) acriflavine. Because of their clonal distribution some or even all of the segregants of any one culture of the heterozygote may be of common origin. For the analysis of segregants of certain independent origin, separate platings should be made, using in each case a different culture derived from a known heterozygous conidium. Only one resistant segregant should be taken from each plating. The selection method described above for the semi-dominant alleles *ACR1* and *ACR3* cannot be applied to the recessive allele *acr2*. No concentration of acriflavine has been found which is sufficiently low to permit enough growth of the *acr2*/+ type for segregation to occur and yet is sufficiently high to select resistant segregants. However, this difficulty can be overcome by first growing the heterozygous types on medium without acriflavine. When growth is sufficient for segregation to have occurred acriflavine is added and the resistant segregants are selected; the technique is as

follows. Conidia of the heterozygote *acr2*/+ are plated so that each plate (diameter 9 cm.) has about 10 conidia suspended in 7 ml. of CM. When this layer is set a further layer of CM (5 ml./plate) is added. The dishes are then incubated for 24 hr., by which time the colonies are 4–8 mm. in diameter. A top evenly-poured layer (8 ml./plate) of CM containing 0.0013% (w/v) acriflavine is then added and incubation continued. In the event that a conidium with a nucleus of genotype *acr2* or *acr2/acr2* was plated the whole colony fairly quickly grows through the acriflavine layer. Such colonies should be ignored as they represent segregation in the previous generation. In the case of a colony which originates from a heterozygous nucleus resistant sectors are seen after 2–4 days of further incubation. The sectors first appear as dense patches of growth (Pl. 1, fig. 3) some of which pierce the acriflavine layer. Most colonies show many sectors of clear clonal distribution; large sectors have their origin near the centre of the colony while the smaller sectors start from nearer the edge. After 6–8 days some of the sectors reach the surface and form conidia (Pl. 1, fig. 4). This technique has not yet been extensively applied and it is not excluded that a proportion of the sectors may arise by mutation. However, haploid segregants of expected genotype and diploid segregants have been obtained, and it seems likely that segregation and not mutation gives rise to the majority of sectors.

DISCUSSION

McIlwain (1941) showed that inhibition of bacterial growth by acriflavine was annulled by nucleotides with which acriflavine forms complex salts. He suggested that acriflavine inactivates enzyme systems of which nucleotides are an essential part. Ephrussi, Hottinguer & Chimènes (1949) and Slonimski & Ephrussi (1949) showed the inhibition by acriflavine of a particle-borne cytochrome system of yeast. The means by which gene-determined resistant strains arise is not yet known. In so far as nucleic acid or its components increases the acriflavine tolerance of both sensitive and resistant strains of *Aspergillus nidulans* the association between acriflavine inhibition and nucleic acid is again confirmed. These resistant strains provide a means for further investigation of the inhibitory action of acriflavine.

The semi-dominant alleles *ACR1* and *ACR3* determine qualitatively similar but quantitatively different phenotypes; they are an example of either allelic or closely-linked mutations which determine similar but not identical phenotypes. It is easily possible to make quantitative estimates of the degree of dominance or recessivity of each of the three mutant alleles and it should be possible to follow dominance modification under conditions favouring such modification.

For the present, the resistant strains have been exploited mainly for use in the parasexual cycle. The semi-dominant and recessive alleles provide a technique for the automatic selection of vegetative segregants of independent origin. The rate of sectoring of heterozygotes gives a relative, though not absolute, estimate of rates of segregation and opens a way for the investigation of genetical and environmental factors which affect this rate. For use in the parasexual cycle the ideal would be to have at least one distal mutant



J. A. ROPER & E. KÄFER—ACRIFLAVINE-RESISTANT MUTANTS OF *ASPERGILLUS*.
PLATE 1

(Facing p. 667)

allele, which confers resistance to one or other substance, on each chromosome arm of the whole chromosome complement. In the case of fungi which have multinucleate conidia the selection of segregants by means of recessive visible markers is likely to be inefficient since only groups of homokaryotic mutant conidia will be detected. Essentially the same applies to those fungi in which the conidial colour markers are non-autonomous. Selection through resistance as described in this account should provide one means of overcoming this difficulty.

The authors are indebted to Professor G. Pontecorvo, F.R.S., for advice and criticism both during the investigations reported and in the preparation of this account.

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EXPLANATION OF PLATE

- Fig. 1. Growth of the strains *ACR1* (right), *ACR1/+* (left) and wild type (top) on CM (left plate) and CM with 0.005 % (w/v) acriflavine (right plate).
- Fig. 2. Resistant sectors formed by strain *ACR1/+* on CM with 0.0025 % (w/v) acriflavine.
- Fig. 3. Resistant sectors formed by strain *acr2/+* on CM with acriflavine. The sectors have not yet reached the surface of the medium. $\times 3$.
- Fig. 4. A resistant sector from strain *acr2/+* after 7 days of incubation. The sector has reached the surface and formed conidia. $\times 3$.

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The Assay of Influenza Virus Particles by Haemagglutination and Electron Microscopy

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SUMMARY: A comparison of an indirect method of counting virus particles (i.e. the 'absolute assay' haemagglutination method) with direct electron-microscope counts showed that the indirect method underestimated the total virus particle count by a factor of about 10. Details of technique and possible sources of error in making counts are discussed.

There has been in recent years increasing interest in quantitative studies of influenza virus, and for this reason accurate and simple ways of assaying the virus have been sought. It is agreed that the haemagglutination titre given by the pattern test is in many respects a stable and accurate index of the amount of virus in a preparation. Published data based on electron-microscopic counts show that there is about one virus particle per red cell in mixtures of virus and red cells which show partial agglutination with this test (Werner & Schlesinger, 1954; Donald & Isaacs, 1954; Isaacs & Donald, 1955).

A modification of the haemagglutination test has been introduced under the name of an 'absolute assay' (Levine, Puck & Sagik, 1953). A mixture of red cells and virus is allowed to sediment in a colorimeter tube, and the change of concentration of red cells in the middle of the tube is followed by a simple colorimeter. From the data the number of rapidly settling red cells is calculated. The number of virus particles is calculated on the assumption that the rapidly settling cells represent pairs of red cells held together by one virus particle. The published data indicated that the results of virus particle assays made in this way agreed with electron-microscope counts made by the spray technique. A similar absolute assay technique has been developed independently and used in extensive quantitative studies on the growth of viruses in the allantoic cavity (Horsfall, 1954).

Those workers who used the absolute assay technique reported that 1 or 2 virus particles may represent 1 egg-infective unit, whereas Donald & Isaacs (1954) found 10 particles visible on electron microscopy for each egg-infective unit present and one particle per red cell at the conventional partial agglutination end-point of the pattern test.

The present investigation began when an attempt was made to prepare carefully standardized and highly infective seed for use in a study on neurotropic strains of influenza viruses. Preliminary investigations showed that there was a tenfold difference between estimates of the concentration of influenza virus particles calculated from haemagglutination data by the

methods of the two groups of workers mentioned above. This is also evident from the data given in Table 1 of Levine *et al.* (1953). The methods were then critically examined and it is now concluded that calculations based on the 'absolute assay' method were erroneous.

METHODS

Virus strains. Influenza virus strains WS and NWS (Francis) were supplied by Dr Alice Moore of the Sloan-Kettering Institute, New York (Francis & Moore, 1940). NWS (Stuart-Harris) was a strain passed in eggs and in chick embryo fibroblasts by Professor C. H. Stuart-Harris. MEL and N.MEL (a neurotropic recombinant of NWS (Stuart-Harris) and MEL described by Burnet & Edney, 1951) were supplied by Dr A. Isaacs. KUNZ was an egg-passaged line of influenza A isolated in Sheffield in 1948 and N-KUNZ was a neurotropic strain prepared with NWS and KUNZ by Dr J. C. Appleby (1952).

Buffered saline. Sodium chloride (0.85 %, w/v) in 0.01 M-phosphate buffer (pH 7.2).

Red cells. Washed hen red cells were suspended in buffered saline and used within a few days.

Propagation of viruses. The seed virus was diluted 10^{-4} or 10^{-5} and 0.2 ml. inoculated to the allantoic cavity of 10- or 11-day chick embryo. Groups of eggs were chilled at 4 hr. intervals and the pooled fluids from each group titrated. The first pool which showed a high HA (haemagglutination) titre was subdivided and stored at -70° .

Egg infectivity titrations. Groups of 4 eggs were inoculated with 3.16-fold (half log) serial dilutions in chilled buffered saline. The eggs were chilled 2 or 3 days later and the fluids tested for the presence of haemagglutinins. Infectivity end-points were calculated by the method of Reed & Muench (1938).

Haemagglutination titrations were carried out by the W.H.O. pattern method, the red cell concentration being obtained from direct counts on the suspension used.

Red-cell sedimentation method. Dilutions of virus were added in volumes of 0.5 ml. to standardized colorimeter tubes of 13 mm. diameter. Ten or 9.5 ml. of 0.5 % (v/v) washed hen red cells was added. The tubes were mixed by inversion and placed under cover. The optical density was recorded on an EEL colorimeter with a green filter. The light beam passed about 35 mm. below the meniscus. Readings were made at 10-30 min. intervals from about $1\frac{1}{2}$ to $4\frac{1}{2}$ hr. after the beginning of the experiment. Occasionally the distance which the cell boundary had dropped below the meniscus was measured with a Perspex ruler.

RESULTS

Features of the red-cell sedimentation procedure

Some general features of the results of sedimentation experiments are shown in Table 1 and Fig. 1 which are abstracted from the records of the titration of a pool of WS virus. This experiment was one of several carried out with the

object of showing whether the sedimentation test behaved as would be anticipated from the theory of Levine *et al.* (1953) and Horsfall (1954). An effect on the rate of sedimentation is seen in the tubes with the first four dilutions (1/10–1/80) of virus. In the first two of these tubes (1/10, 1/20) all the cells were settling rapidly since the boundary was descending faster than the controls. However, in the dilutions 1/40 and 1/80 the optical density was decreased but the boundary was descending as in the controls. In tubes such as these it is possible to assume that there is a mixture of single cells and rapidly settling agglutinated cells, and over the narrow range covered there appears to be direct proportionality between the amount of rapidly settling cells (Table 1, optical density column *g* minus optical density column *c* or *d*) and the amount of virus added.

Table 1. *Observations on mixtures of hen red cells and influenza A virus, strain WS, in colorimeter tubes. The relation of the increased rate of sedimentation of cells in the presence of virus to the agglutination pattern of sedimented cells*

Measurement made	Time (min.)	Dilution of virus						Saline control <i>g</i>
		1/10 <i>a</i>	1/20 <i>b</i>	1/40 <i>c</i>	1/80 <i>d</i>	1/160 <i>e</i>	1/320 <i>f</i>	
Optical density	0	0.56	0.57	0.57	0.57	0.57	0.58	0.58
Optical density	124	0.15	0.23	0.33	0.43	0.48	0.49	0.49
Distance boundary sedimented (cm.)	135	2.8	2.4	2.3	2.2	2.2	2.3	2.3
Optical density	205	0.04	0.04	0.08	0.16	0.26	0.24	0.24
Distance boundary sedimented (cm.)	207	6	4	3.5	3.5	3.3	3.3	3.5
Agglutination pattern of sedimented cells	.	+++	+++	+++	++	0	0	0

The proportion of rapidly settling cells was usually derived from a graph such as that in Fig. 1, in which a straight line was fitted by eye to points obtained by plotting optical density against time. It appeared that there were irregularities in the settling process in the early and late phases, and one rarely saw a clear-cut phase demonstrating the rapid settling of agglutinated cells in tubes containing virus. However, recordings made using the intermediate stages were reasonably consistent, and at times the settling curves suggested the pattern of converging lines expected theoretically (Fig. 1). Levine *et al.* (1953) mentioned curves of settling of virus+red-cell mixtures which were parallel to the control curve. This would not be expected from their theory since, if the boundary of single cells were descending at the same rate in the control and in the experimental tubes, they would pass through the light path at the same time and the optical density would reach zero at the same time in both tubes. If the curves were parallel, the ratio of sedimented to unsedimented cells would vary from point to point along the curves.

At the beginning of this experiment a 0.25 ml. sample was removed from each tube and allowed to form a pattern in a Perspex plate. The result is shown

in the bottom row of Table 1 and indicates that the last mixture of red cells and virus which showed accelerated sedimentation also showed partial agglutination by the pattern method (i.e. represented the haemagglutination end-point). A similar relationship between the end-points was noted in experiments with KUNZ, N-KUNZ, NWS (Francis), NWS (Stuart-Harris), MEL and N.MEL. (Egg infectivity titrations were carried out on all these fluids and the average ratio of EID₅₀ to 'absolute assay' particle counts was 1.3.) That

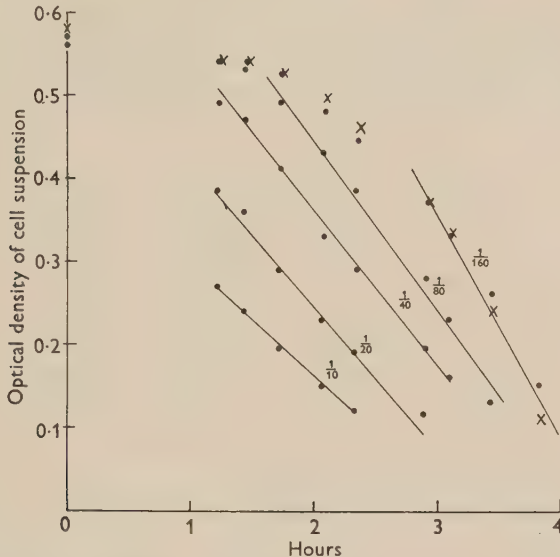


Fig. 1. Photoelectric colorimeter readings across the middle of 6 tubes during the sedimentation of mixtures of red cells and various dilutions of WS virus. \times — \times indicates the control mixture without virus; \bullet — \bullet indicates mixture virus + red cells. The initial dilution of virus is shown by each curve. More details are given in the text and in Table 1.

partial haemagglutination in the pattern test and an increased sedimentation rate in the 'absolute assay' should occur at the same dilution of virus indicates clearly that one of the methods of estimating the number of virus particles from haemagglutination data must be wrong. From the data of Donald & Isaacs (1954) we should expect, at the pattern-test end-point, 1 virus particle for each red cell present in the mixture. But from the 'absolute assay' data on this same mixture Horsfall (1954) or Levine *et al.* (1953) would calculate that 33% of the cells are settling rapidly in doublets, and that therefore the number of virus particles is half 33% of the number of red cells, i.e. about 1 particle for each 6 cells present.

The relation between direct particle counts, standard haemagglutination titrations and the sedimentation method

An experiment was therefore carried out to determine the number of particles at the haemagglutination end-point for 5 different strains by counting the virus particles directly with the electron microscope by two techniques,

the red-cell absorption method (Dawson & Elford, 1949; Donald & Isaacs, 1954) and the spray-drop method (Williams & Backus, 1949). Seeds of WS, NWS and MEL were diluted 10^{-4} , seeds of KUNZ and N.KUNZ were diluted 10^{-3} . Nine-day eggs were inoculated with 0.2 ml. of these dilutions and the allantoic fluids harvested 24 hr. later. For each virus one clear fluid which gave a positive haemagglutination test was selected for study.

To determine the count by the red-cell method, a measured volume of the virus fluid was mixed with a known number of washed, saponin-lysed hen red cells (ghosts). The virus particles were almost completely adsorbed on these ghosts, the supernatant fluids in all cases showing less than 2% residual haemagglutinating activity. The mean number of particles/ghost was then found by electron microscopy. The only departure from the techniques described by Donald & Isaacs (1954) was in counting virus particles on the upper and lower surfaces of each ghost as we were unable to distinguish the particles adsorbed on the top of the membrane. This is illustrated by Pl. 1. Pl. 1, fig. 1, shows a ghost before the preparation was metal-shadowed; there is sufficient contrast to identify the adsorbed virus particles and there can be no difference between those on the upper and lower surfaces of the membrane. Pl. 1, fig. 2, shows this ghost after metal-shadowing; the same particles are all to be seen and there is no clear distinction between their shadows to show which lie on top. Presumably the cell membrane is so thin that the particles on the underside push through it as the preparation dries down. Clear counts on all five virus fluids were made by this method.

Counts by the spray-drop method were made after dialysing 0.5 ml. of each virus fluid against 100 vol. distilled water for 18 hr. Haemagglutination titrations on the dialysed fluids did not differ significantly from those made before dialysis, and the fluids were then mixed with known numbers of polystyrene latex spheres and sprayed on to prepared grids without delay. The details of these techniques were as described in Luria, Williams & Backus (1951). Some difficulties occurred in making these counts. By placing the fluid as harvested directly on specimen grids and examining them in the electron microscope, it was noticed that one strain (KUNZ) consistently gave fluids containing much debris in addition to the virus particles (Pl. 2, fig. 3), while all the other strains gave fluids almost entirely free from debris. Pl. 2, fig. 4, shows the appearance of the WS fluid. During dialysis of the KUNZ fluid, much of the debris precipitated; the spray drops, however, were filled with many tiny particles (Pl. 2, fig. 5). Very few typical virus particles were found in these drops, although, as already stated, the haemagglutination titre showed no decrease during the dialysis. For this reason spray counts were not made with the KUNZ strain; the NWS fluid was also unsuitable on account of its low titre. The other three strains gave satisfactory counts; Pl. 2, fig. 6, shows part of a spray drop of MEL.

The dispersion of particles in the spray drops was improved by more complete dialysis than that described, but it was found that the virus particles were unstable in low salt concentrations. Thus on dialysing MEL for 4 days against two changes of water, the haemagglutination activity decreased more

than 50-fold to a titre of less than fifty. Despite this loss of titre, virus particles were still identifiable though some showed degenerative changes.

The results of these experiments are summarized in Table 2, which shows that there was reasonable parallelism between the results of all four measurements on the various fluids. The two methods of electron microscopy gave very similar particle counts, but these were 6- to 20-fold higher than those estimated by the 'absolute assay' procedure.

Table 2. *Measurements on five allantoic fluids containing influenza virus. The discrepancy between the numbers of virus particles determined by electron microscope counts and by the red-cell sedimentation ('absolute assay') procedure*

Strain	HA units* per ml.	'Absolute assay' particles per ml.	Electron microscope particle counts		Ratio:† EM particles HA
			Red-cell method per ml.	Spray method per ml.	
WS	5200	8.5×10^9	53×10^9	60×10^9	1.1×10^7
NWS	200	0.80×10^9	5.5×10^9	—‡	2.75×10^7
KUNZ	1000	2.3×10^9	14×10^9	—‡	1.4×10^7
N. KUNZ	400	0.88×10^9	18×10^9	18×10^9	4.5×10^7
MEL	2000	3.8×10^9	24×10^9	33×10^9	1.4×10^7

* One HA unit gives partial agglutination with an equal vol. of 1 % (v/v) hen red cells (2.5×10^7 cells/ml.).

† Ratio = particles by electron microscope count/haemagglutination units.

‡ Too few particles (NWS) or too much debris (KUNZ) to do accurate counts.

The ratio particles : haemagglutination units range from 1.1×10^7 to 4.5×10^7 for the electron microscope counts on the various strains and, since the count of the red-cell suspension used was 2.5×10^7 , the ratio of virus particles to red cells at the partial agglutination end-point was about 1, as previously found with other influenza A strains (Donald & Isaacs, 1954).

In other experiments of this sort in which similar results were obtained a WS fluid was studied which had an infectivity titre of $10^{9.7}$ and an 'absolute assay' particle count of $10^{9.9}$. It could therefore be called a 'fully infectious fluid' (Horsfall, 1954), but the electron microscope count was $10^{11.0}$ showing that, despite agreement between the two titres, neither was a true measure of the number of virus particles present.

DISCUSSION

These experiments force upon us a very cautious and empirical attitude toward the deduction of virus particle counts from haemagglutination data. It seems that the haemagglutinating virus 'absolute assay' method is based on an unjustified assumption. The data indicate that either one virus particle cannot tie together two red cells, or perhaps, if it can do so, it has about a one in ten chance of doing so during the conduct of the experiment. It seems the mumps virus and influenza C virus particles are even less efficient in producing

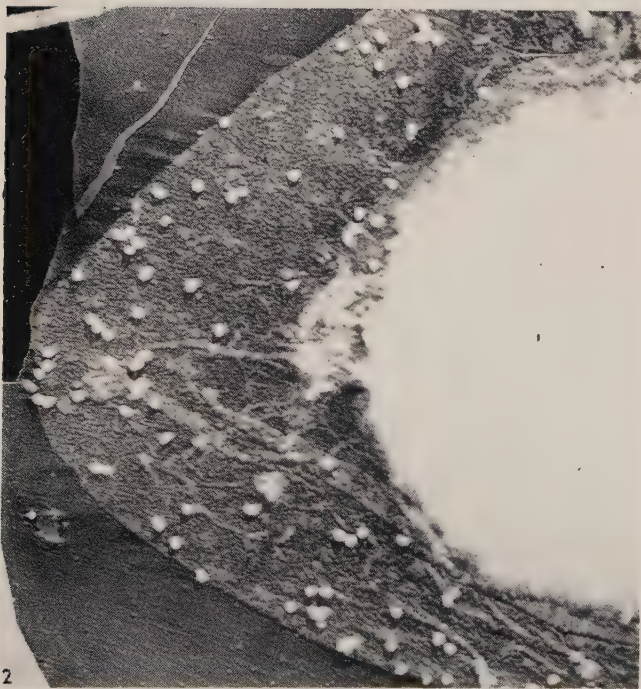
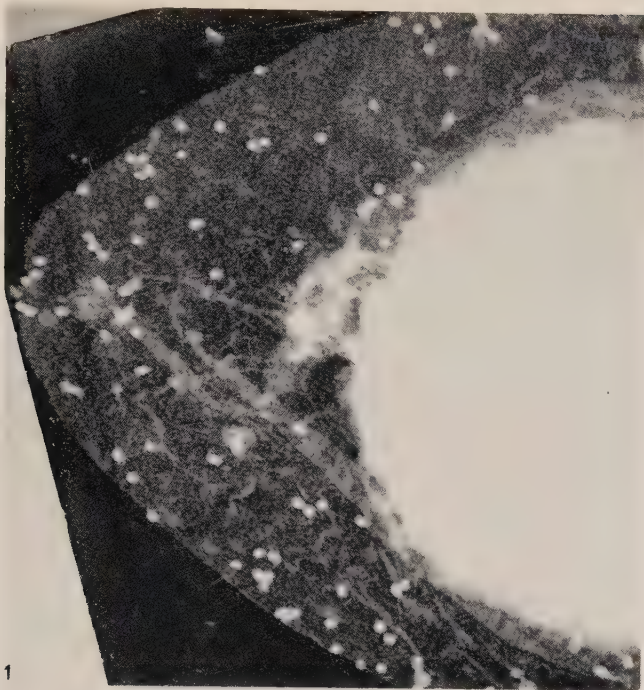
haemagglutination since they are present in two- or threefold higher concentration than influenza A particles at the agglutination end-point (Isaac & Donald, 1955). There is suggestive evidence in our data that the particles of neurotropic strains of influenza virus may be less efficient than their parent strains in inducing haemagglutination. These differences may reflect differing affinities of the virus for red-cell receptors.

It is difficult to explain why Levine *et al.* (1953) obtained apparent confirmation of their theory from electron microscope counts. We note, however, that they used centrifuged and resuspended particles and only used the spray method. We have found that apparently simple manipulations like dialysis and storage may drastically decrease spray counts, and it is possible that technical factors of this sort might have affected the experiments of Levine *et al.*

The data indicate that the 'fully infectious seed' contains in fact many more virus particles than infectious units, but this is a discrepancy found generally among viruses (Luria, 1953), perhaps because not all particles reach susceptible cells or, if they do so, fail to initiate infection. These factors operate even when precautions are taken to avoid thermal inactivation of the virus after release from cells, and of course it is not known whether all virus particles are potentially infectious even immediately after release from a host cell. The data also indicate the need to recalculate and re-interpret work carried out by using the sedimentation techniques. For example, one of us (Tyrrell, 1955) calculated the yield of virus particles from chick embryo lung cells infected with the WS strain. The result given was just under 10^3 particles/cell, whereas it would become 10^4 particles/cell if calculated from either the sedimentation or pattern haemagglutination experiments, using the factors found in this investigation.

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D. A. J. TYRRELL & R. C. VALENTINE—ASSAY OF INFLUENZA VIRUS PARTICLES.
PLATE 1

(Facing p. 674)



D. A. J. TYRRELL & R. C. VALENTINE. ASSAY OF INFLUENZA VIRUS PARTICLES.
PLATE 2

- REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent end points. *Amer. J. Hyg.* **27**, 493.
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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Influenza virus particles adsorbed on a lysed hen red blood cell. Fixed with 0.1 % osmium tetroxide. Unshadowed. $\times 12,000$.
- Fig. 2. The field shown in Fig. 1 after shadowing with gold + palladium; $\times 12,000$.

PLATE 2

- Fig. 3. Influenza virus particles (KUNZ strain) as harvested in allantoic fluid with much cell debris. Fixed with osmium tetroxide vapour and treated with 5 % phosphotungstic acid. $\times 24,000$.
- Fig. 4. Influenza virus particles (WS strain) as harvested. Little debris. Fixed with osmium tetroxide vapour and treated with 5 % phosphotungstic acid. $\times 24,000$.
- Fig. 5. Part of a spray drop of KUNZ fluid after dialysis mixed with latex spheres. It contained unexpectedly few virus particles but many smaller particles. Shadowed with gold + palladium. $\times 14,000$.
- Fig. 6. Part of a spray drop of MEL fluid after dialysis mixed with latex spheres. The virus particles can be clearly identified and counted. Shadowed with gold + palladium. $\times 14,000$.

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Lactobacillus helveticus

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SUMMARY: Two of the *Lactobacillus* species previously defined, *Lactobacillus helveticus* and the 'intermediate group', have been renamed *L. casei* var. *rhamnosus* and *L. helveticus* (Orla-Jensen) Holland, respectively. Antisera prepared against *L. bulgaricus* and *L. helveticus* (Orla-Jensen) Holland show that these species belong to the same serological group. The names of the serological groups previously defined have been replaced by letters.

Much confusion exists concerning the identity of '*Lactobacillus helveticus*'. Orla-Jensen (1919) renamed Freudenreich's *Bacillus casei* ϵ as *Thermobacterium helveticum*. Later (Holland, 1920) this name was amended to *Lactobacillus helveticus* (Orla-Jensen). Orla-Jensen described it as a high-temperature organism, fermenting glucose, fructose, galactose, lactose, mannose, maltose and dextrin, but not mannitol, sucrose, salicin, glycerol, rhamnose, arabinose, xylose, raffinose, inulin or sorbitol. The description of *L. helveticus* (Orla-Jensen) Holland in *Bergey's Manual of Determinative Bacteriology* (1948) agrees with this. Wheeler (1955*a*) described the species as a high-temperature *Lactobacillus* fermenting those same sugars and in addition mannitol, sucrose, salicin, glycerol and rhamnose. This classification was based on named strains of '*L. helveticus*' in our collection. Of 21 such strains 3 were identified (Wheeler, 1955*a, b*; Sharpe, 1955) as *L. casei*, 2 as *L. bulgaricus*, 4 as 'intermediate group' (intermediate between *L. acidophilus* and *L. bulgaricus*), 2 as *L. lactis*, and the remaining 10 strains formed the nucleus of our group '*L. helveticus*'. These organisms were all strains labelled *L. helveticus* when received from other workers and from national type collections; none was a fresh isolate named only by us. Since then, however, we have learnt in a private communication from Dr R. G. Jensen that the original strain of *L. helveticus* described by Orla-Jensen is still extant, and is indeed one of the strains of lactobacilli in our collection. This strain, *L. helveticus* ATCC 8018, NCIB 8025, was previously identified by Wheeler (1955*b*) as a member of her 'intermediate group'; she regarded this group as being possibly related to *L. bulgaricus*, and had not defined it as a species. The physiological characteristics of this 'intermediate group', including strain ATCC 8018 and 3 other named strains of *L. helveticus* in our collection, agree completely with those of Orla-Jensen's original description of *L. helveticus*. In view of these findings, which are based on the original strain of *L. helveticus*, we propose to rename Wheeler's 'intermediate group' as *L. helveticus* (Orla-Jensen) Holland.

The characteristics of *Lactobacillus helveticus* (Orla-Jensen) Holland are

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given below, and are those of the group previously known as the 'intermediate group', namely:

No growth at 15° or below. Maximum temperature between 45° and 50°. Homofermentative. Ferments glucose, fructose, galactose, mannose, maltose, lactose, dextrin. Does not ferment mannitol, sucrose, salicin, glycerol, inositol, rhamnose, arabinose, xylose, raffinose, inulin, sorbitol, amygdalin, cellobiose, melibiose, melezitose. Grows in the presence of 2% (w/v) but not 4% (w/v) NaCl. Does not grow in 2% (w/v) bile salt.

Wheater (1955a) and Davis (1955) concluded that *Lactobacillus casei* var. *rhamnosus*, described by Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953), and our organism previously designated '*L. helveticus*' were the same organism. Now that we have redesignated our 'intermediate group' as *L. helveticus* (Orla-Jensen) Holland we consider that our strains previously named *L. helveticus* should be reclassified as *L. casei* var. *rhamnosus* as the descriptions of the two agree so nearly. It was suggested previously that this variety differs sufficiently from *L. casei*, i.e. in its ability to grow at 45° and to ferment rhamnose, sorbitol and inositol, to justify giving it species rank. However, we have since isolated several strains of *L. casei* which, although they were able to grow at 45°, were not var. *rhamnosus*, and the separation of *L. casei* var. *rhamnosus* as a new species solely on these few sugar fermentations is not considered justified. *L. casei* and *L. casei* var. *rhamnosus* belong to the same serological group (Sharpe, 1955), but strains having the physiological characteristics of *L. casei* var. *rhamnosus* usually possess a specific type antigen (Orland, 1950; Sharpe, 1955).

Further confusion is attached to the *Lactobacillus* strain ATCC 7469 which for many years has been used extensively for microbiological assay work and widely issued for this purpose. Tittsler, Rogosa & Whittier (1942) and Orland (1950) described how this organism became known variously as *L. casei* ϵ , *L. casei* and *L. helveticus*; Tittsler *et al.* (1942) also showed that it was a strain of *L. casei* which could be distinguished from *L. helveticus* by physiological characters, and the name of the culture was then amended by the American Type Culture Collection to *L. casei*. Since then it has been further identified as *L. casei* var. *rhamnosus*. It appears, however, from a study of the many named cultures sent to us as *L. helveticus*, but which were in fact *L. casei* var. *rhamnosus*, that strains of this organism, or of organisms identified by the same characteristics, and labelled *L. helveticus* are still to be found in many collections. We possess both *L. casei* ATCC 7469 and *L. helveticus* ATCC 7469. Two of the strains received by us, namely ATCC 11981 (NCIB 6557) and ATCC 11982 (NCIB 8103) which are at present listed in both the American Type Culture Collection and the National Collection of Industrial Bacteria catalogues as *L. helveticus*, are in fact *L. casei* var. *rhamnosus*. It is therefore suggested that workers who include strains labelled '*L. helveticus*' in their culture collections might be wise to check the physiological properties of these organisms. *L. casei* var. *rhamnosus* ferments mannitol, sucrose, salicin, glycerol, inositol, rhamnose, cellobiose and melezitose, grows at 15° and hydrolyses aesculin; *L. helveticus* (Orla-Jensen) Holland has none of these properties.

Serological position of Lactobacillus helveticus

Further work has now shown that *Lactobacillus helveticus* (Orla-Jensen) Holland is a member of the serological group *L. bulgaricus*. Using methods previously described (Sharpe, 1955), a more potent antiserum against *L. bulgaricus* has now been prepared. This antiserum reacted by precipitin test with HCl extracts of all strains previously classified as *L. bulgaricus*, and in addition with extracts of all 15 of the strains now physiologically identified by us as *L. helveticus* (Orla-Jensen) Holland. A serum has also been prepared against a strain of *L. helveticus*, which has been found to react with extracts of *L. bulgaricus* strains as well as with those of *L. helveticus* strains, so that

Table 1. *Proposed nomenclature for serological groups of lactobacilli*

New designation	Former designation*	Species included in group
Group A	<i>L. bulgaricus</i>	<i>L. bulgaricus</i> and <i>L. helveticus</i>
Group B	<i>L. casei</i>	<i>L. casei</i>
Group C	<i>L. casei-helveticus</i>	<i>L. casei</i> and <i>L. casei</i> var. <i>rhamnosus</i>
Group D	<i>L. plantarum</i>	<i>L. plantarum</i>
Group E	<i>L. lactis-brevis</i>	<i>L. lactis</i> , subgroup <i>L. brevis</i>
Group F	<i>L. fermenti</i>	<i>L. fermenti</i>

* As given in Sharpe (1955).

these two species belong to the same serological group. It was observed, however, that each species reacted somewhat differently with the group antisera. Extracts of strains of *L. bulgaricus* (including different serological types) generally gave a stronger reaction with *L. bulgaricus* antiserum and a weaker reaction with *L. helveticus* antiserum, whereas extracts of *L. helveticus* gave a stronger reaction with *L. helveticus* antiserum and a weaker one with *L. bulgaricus* antiserum. There appeared to be a qualitative difference here. Differential absorption tests were attempted but neither of the antisera, particularly the rather weak *L. helveticus* one, were potent enough to give clear-cut results; any absorption, even with heterologous strains, greatly weakened the precipitin reactions. Although the two species belong to the same serological group, these differences in degree of reaction suggest some difference in antigenic components.

Other workers (Dr R. G. Jensen, private communication; Davis, 1955) have suggested a close relationship between these two species and the serological results bear this out. Further work on the characteristics of the two groups is still needed before it can be decided whether *Lactobacillus helveticus* (Orla-Jensen) Holland deserves species rank or whether it should be regarded as a variety of *L. bulgaricus*. Until then we prefer to retain its specific rank.

Nomenclature of the serological groups of lactobacilli

We would like to take this opportunity of renaming the serological groups of lactobacilli by letters instead of by the names of the physiologically defined species contained in them. The work described above confirms our previous

findings (Sharpe, 1955) that a serological group may contain more than one species, and the present method may become very cumbersome. We propose, therefore, to name the serological groups as shown in Table 1. This should eliminate any confusion which might arise between the serological and physiological methods of classification and allow for the definition of further species within a serological group. It has not yet been possible to prepare a serum against *Lactobacillus acidophilus*, which has been physiologically defined as a species.

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The Structure of Viruses of the Newcastle Disease-Mumps-Influenza (Myxovirus) Group

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SUMMARY: Particles of influenza, mumps, fowl plague, Newcastle disease and Sendai viruses were adsorbed on electron microscope films and treated with acid, trypsin and ribonuclease. All of these viruses contained trypsin-resistant rings of ribonucleoprotein and with some strains these rings showed lines of staining which may indicate the arrangement of the nucleic acid.

We recently described an investigation of the structure of influenza A virus by a new technique (Valentine & Isaacs, 1957). The virus particles were adsorbed on to the nitrocellulose film covering an electron microscope specimen support, treated with proteolytic enzymes and other reagents *in situ* and examined directly in the microscope. By this method it was shown that influenza virus spheres contain a ring of ribonucleoprotein enclosed within a protein coat; the arrangement of the carbohydrate and lipid in the particles is not known. This result contrasts with the structure of the pox viruses shown in the work of Dawson & McFarlane (1948), Peters & Stoeckenius (1954) and others where a central solid nucleus-like structure of deoxyribonucleoprotein is enclosed within a protein coat. We have now investigated other members of the *Myxovirus* group, and all those which we have studied have contained a trypsin-resistant ring of ribonucleoprotein. In some cases these rings themselves have shown remarkable structural details.

METHODS

Virus strains. Newcastle disease virus: 'Herts' strain; Vaccine 'B' strain supplied by Dr F. B. Bang. Sendai virus (Kuroya, Ishida & Shiratori, 1953) supplied by Dr W. W. Ackermann. Mumps virus: Enders strain, EMA 41. Fowl plague: Brescia strain. Influenza B: LEE, 1940 and Crawley, 1946 strains. Influenza C: the original 1233 strain of Taylor (1949).

Preparation of viruses. Newcastle disease and fowl plague viruses were inoculated into the allantoic cavity of 10-day chick embryos and harvested after 18-24 hr. at 37°. Sendai and influenza B viruses were similarly inoculated, but harvested after 2-3 days of incubation. Mumps virus was inoculated into the allantoic cavity of 8-day eggs and the fluids harvested 5 days later. Influenza C virus was inoculated amniotically into 10-day eggs, and the allantoic fluids harvested after 2-3 days of incubation. In some experiments virus was adsorbed on to chick red cells and eluted into saline, but in most experiments virus in the allantoic fluid was used directly for electron microscopy after light centrifugation only.

Other techniques. Other materials and the techniques of electron microscopy were described in detail in our previous communication (Valentine & Isaacs, 1957); the following is a brief description of the methods used. A drop of the virus preparation to be examined was placed on a nitrocellulose film on a platinum support for 1 min. and then washed away by immersing it in distilled water. The specimen support with many virus particles adsorbed on its film was then placed in 0.1 N-HCl for 2 min., washed and incubated in 0.1 % (w/v) crystalline trypsin at pH 8.0 for 1 hr. at 37°. The pre-treatment with acid was essential for digestion and probably served to denature the protein. In some experiments the digested particles were examined after the trypsin treatment, but in most experiments the films were subsequently immersed in 0.1 % (w/v) crystalline ribonuclease at pH 8.0 for 1 hr. at 37°. After treatment with these enzymes, the structures on the films were fixed in osmium tetroxide vapour, treated in 5 % (w/v) phosphotungstic acid and washed in distilled water. No drying of the films took place until after this final wash. Control preparations of the same virus material were examined in the electron microscope without treatment with enzymes but with the same fixation with osmium tetroxide and subsequent treatment with phosphotungstic acid.

The regions where the phosphotungstic acid is taken up by the specimen scatter electrons and appear electron-dense; they are reproduced dark in the Plates. Such treatment has usually been referred to by electron microscopists as 'staining' by analogy with the use of the word in light microscopy to describe treatment with a coloured pigment. The word 'staining' will be used here to mean the effect of treatment with phosphotungstic acid in rendering parts of the specimen specifically electron-dense.

RESULTS

After treatment with acid and trypsin, the viruses of the Myxovirus group all showed rings of trypsin-resistant material similar to those already described for influenza A (Valentine & Isaacs, 1957). The rings had often expanded during the digestion and were, as a rule, slightly larger than the particles from which they had come. These rings took up very little phosphotungstic acid and were rather vague and ill-defined. Pl. 1, fig. 1, shows a Newcastle disease virus particle after HCl and trypsin treatment. However, when this treatment was followed by further treating the films with ribonuclease, the rings, still undigested, now 'stained' more intensely, often showing striking structural detail (Pl. 1, fig. 2). The ring structures were completely digested by following the ribonuclease with further HCl and trypsin treatment. But this complete digestion of the particles was only obtained by using the full sequence of treatments; HCl, trypsin, ribonuclease, HCl and trypsin, carried out in that order. Thus, as in the case of influenza A (Valentine & Isaacs, 1957), the rings can reasonably be identified as ribonucleoprotein structures contained within the intact virus particles.

Ring structures were prepared from the different viruses by treating virus

particles adsorbed on the supporting film with acid, trypsin and ribonuclease, followed by fixing and 'staining'. Pl. 1, figs. 2-4, shows rings obtained from two strains of Newcastle disease, and Pl. 1, fig. 5, one from Sendai virus. The general appearances of the rings from these three viruses were very similar. In many places 3 to 6 evenly spaced dark lines of 'staining' could be seen running along the rings. These lines were broken at points but they seldom appeared to cross one another. When instead of being treated with phosphotungstic acid, the rings were shadowed with platinum (Pl. 2, fig. 6) the length of the shadows suggested that the rings were about as high as they were thick, i.e. that they might well have been circular in cross-section.

Plate 2, fig. 7, shows a ring typical of those obtained from mumps virus; lines of 'staining' were not clearly seen. Fowl plague virus, on the other hand, showed rings with 3 or more lines (Pl. 2, fig. 8). Some of the rings from the LEE strain of influenza B were similar but with less distinct lines (Pl. 2, fig. 9); others showed no detail. No detail was seen in the rings of the Crawley strain of influenza B nor in those of influenza C (Pl. 2, fig. 10).

DISCUSSION

Two facts of interest have emerged from this investigation. First, all the viruses of the Myxovirus group which we examined showed trypsin-resistant structures in the form of rings apparently composed of ribonucleoprotein. Secondly, after treatment with ribonuclease the ring structures frequently showed characteristic dark lines of 'staining' most strikingly seen with Newcastle disease virus. This 'staining' effect can be explained in one of two ways. The ribonuclease might have removed threads of ribonucleic acid from the surface of the rings and exposed basic groups with a strong affinity for phosphotungstic acid in the underlying protein. Alternatively, the ribonuclease might have remained combined with the nucleic acid and itself taken up phosphotungstic acid. Though the first explanation seems the more probable, there is no conclusive evidence in favour of either. However, whichever is correct, it would appear that the dark lines of 'staining' may well indicate the arrangement of the ribonucleic acid in the ring structures.

It was earlier suggested (Valentine & Isaacs, 1957) that the treatment with HCl necessary before the trypsin had any marked effect on the virus particle served to denature the protein and thus render it susceptible to the enzyme. If this is so, the fact that a second treatment with HCl was needed before trypsin could digest away the ribonuclease-treated ring has some significance. It suggests that the first treatment with HCl denatured all the protein in the virus particle except that in the nucleoprotein ring. This could only be denatured after the nucleic acid in the ring had been attacked by ribonuclease. The nucleic acid must thus confer considerable structural stability on the protein that forms the bulk of the ring. This stability is apparently often combined with some rigidity, for in the intact particle the rings would have been orientated at random with respect to the supporting film and become adsorbed on to it as the rest of the particle was digested away.

Nevertheless, at least with Newcastle disease and Sendai viruses, the rings normally appeared as regular structures despite the many distorting forces which must have acted on them.

The actual arrangement of the dark lines of 'staining' is of particular interest if they can in fact be identified with the position of the nucleic acid in the virus. It seems clear that the lines must represent threads rather than lamellae for it would be extremely unlikely that lamellae so closely spaced would so often lie parallel to the electron beam. If threads, then it is probable

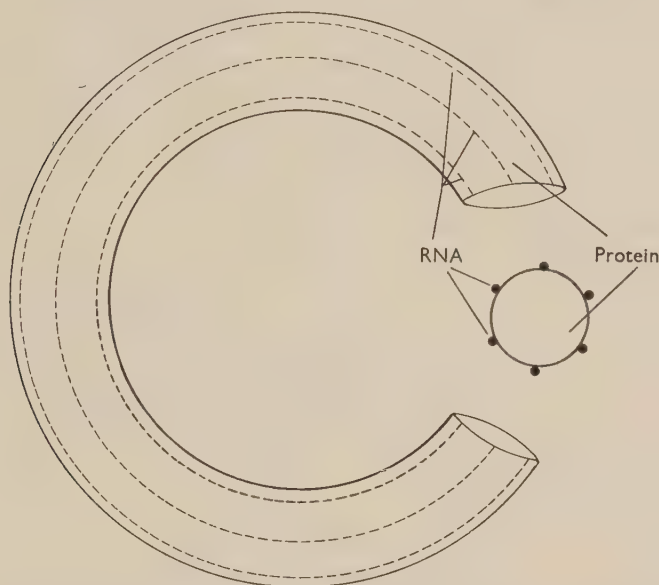


Fig. 1. Proposed arrangement of ribonucleic acid (RNA) and protein in ribonucleoprotein ring of Newcastle disease virus.

that they lie on the surface of the ring since one line at least often seems to run along an edge. Some of the dark lines are denser and thicker than others and in places can be seen to be double (e.g. Pl. 1, fig. 4). The most likely interpretation is that here two lines are being viewed almost superimposed, one being on the top and one on the bottom of the ring.

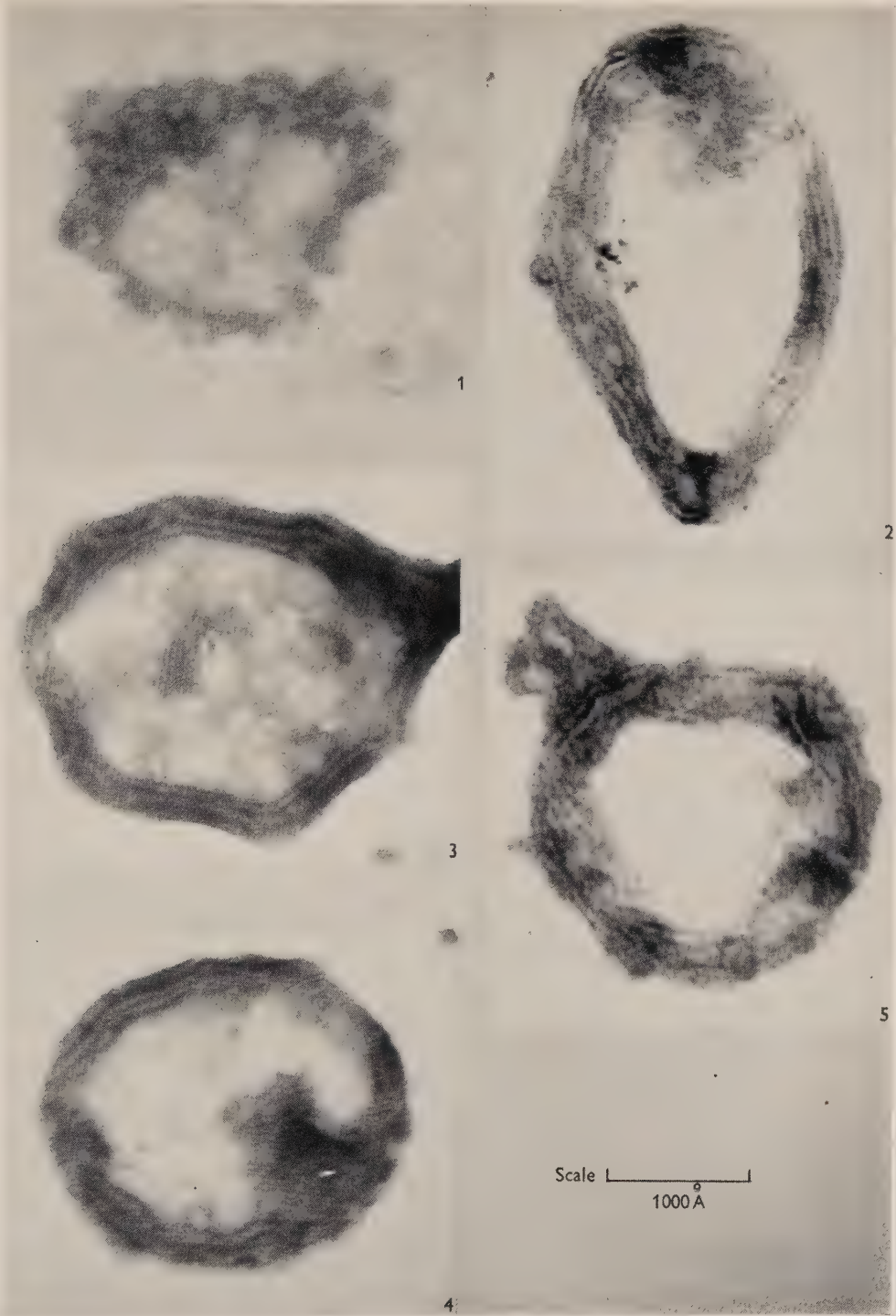
In those viruses that have shown well-defined lines of 'staining', in particular those of Newcastle disease and Sendai, we therefore picture the nucleoprotein as a ring consisting of a protein core stabilized by threads of ribonucleic acid running along its surface as shown in Fig. 1. This has been drawn with 6 threads but the number that would be seen on an electron micrograph of such a structure could vary between 3 and 6 depending on whether they were superimposed in the image. The essential feature of our model is the general arrangement of the nucleic acid and protein and not the actual number of nucleic acid chains which cannot be deduced with any certainty from the pictures. In the intact spherical particle we suppose the ring of nucleoprotein to lie just within the surface, embedded in the other components of the virus.

The influenza and mumps viruses whose rings have not clearly shown similarly arranged dark lines of 'staining' may have a different arrangement of their nucleoprotein. On the other hand, these lines may for some reason merely be more difficult to reveal.

Frisch-Niggemeyer (1956) pointed out that, despite their varying sizes, all the viruses which contain ribonucleic acid and for which reliable chemical analyses are available appear to contain roughly the same mass of nucleic acid per particle, corresponding to about 6000 nucleotides. Little is yet known about the detailed structure of ribonucleic acid, particularly as it exists within the virus. If the molecules were fully extended structures the distance between the nucleotides would be about 7.5 Å. and thus the mean length of ribonucleic acid in each virus about 45,000 Å. However, if the structure is not fully extended but coiled in some way as is deoxyribonucleic acid (Crick & Watson, 1954) the length of the nucleic acid would, of course, be considerably less than 45,000 Å.; in the double helix of deoxyribonucleic acid, for example, 20 nucleotides correspond to a length of 34 Å. and so 6000 nucleotides would be contained along a structure only 10,000 Å. long. In fact, the actual total length of the lines of 'staining' seen in the Newcastle disease virus rings (Pl. 1, figs. 2-4) has varied from *c.* 10,000 to 30,000 Å. per ring. Thus these rough estimates at least serve to show that there is no obvious inconsistency between the suggested structure as deduced from the electron micrographs and what is at present known chemically about the nucleic acid content of the virus particles.

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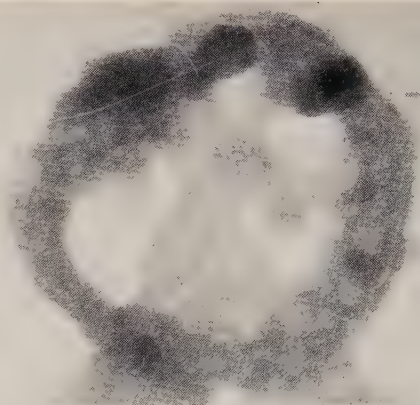


R. C. VALENTINE & A. ISAACS—VIRUS STRUCTURE. PLATE 1

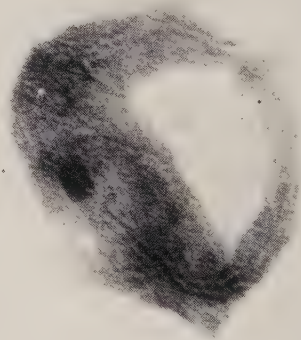
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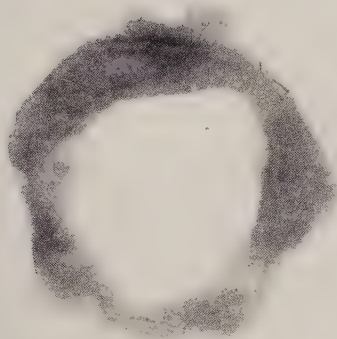
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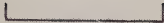
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EXPLANATION OF PLATES

PLATE 1

The effect of treating the virus particles with 0.1 N-HCl and 0.1 % trypsin (fig. 1) and 0.1 N-HCl, 0.1 % trypsin and 0.1 % ribonuclease (figs. 2-5). The treated preparations were fixed with osmium tetroxide vapour and then treated with phosphotungstic acid. All magnifications $\times 210,000$.

Figs. 1, 2. Newcastle disease virus ('Herts' strain).

Fig. 3. Newcastle disease virus (Vaccine B strain).

Fig. 4. Newcastle disease virus ('Herts' strain).

Fig. 5. Sendai virus.

PLATE 2

The effect of treating the virus particles with 0.1 N-HCl, 0.1 % trypsin and 0.1 % ribonuclease. The treated preparations were fixed with osmium tetroxide vapour and either shadowed with platinum at 30° (fig. 6) or treated with phosphotungstic acid (figs. 7-10). All magnifications $\times 210,000$.

Fig. 6. Newcastle disease virus ('Herts' strain).

Fig. 7. Mumps virus.

Fig. 8. Fowl plague virus.

Fig. 9. Influenza B virus.

Fig. 10. Influenza C virus.

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Metabolism of Methoxylated Aromatic Compounds by Soil Fungi

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SUMMARY: The metabolism of methoxylated aromatic compounds by the soil fungi *Haplographium* sp., *Hormodendrum* sp. and *Penicillium* sp. has been investigated. A study of rates of decomposition of mono-methoxybenzoic acids by *Hormodendrum* sp. revealed that they are most rapidly attacked in the order *para* (*p*), *meta* (*m*) and *ortho* (*o*). In respiration studies with all three fungi the *p* form was again found to be metabolized most rapidly. In the initial stage of attack the methoxyl group is replaced by a hydroxyl group. *Penicillium* sp. also formed *p*-methoxyphenol from *p*-methoxybenzoic acid. A study of the rates of metabolism of monohydroxybenzoic acids revealed that they are attacked in the same order as the monomethoxybenzoic acids. *p*-Hydroxybenzoic acid formed from *p*-methoxybenzoic acid is further metabolized to protocatechuic acid by *Hormodendrum* sp. and *Penicillium* sp. When veratric acid (3:4-dimethoxybenzoic acid) is incubated with *Hormodendrum* sp. and *Penicillium* sp. the methoxyl group in the *p* position is replaced by a hydroxyl group to give vanillic acid. All three fungi formed two unidentified phenolic compounds from 2:4-dimethoxybenzoic acid. The possible significance of the results in the decomposition of lignin in soil is discussed.

Previous investigations revealed that a number of soil fungi were capable of utilizing simple lignin-related phenolic compounds as sole source of carbon (Henderson & Farmer, 1955) and some information on the metabolism of these compounds has also been obtained (Henderson, 1956). A feature of the decomposition of lignified material in soil is the significant reduction in methoxyl content (e.g. Sowden & Atkinson, 1949). In the present work a study has been made of the breakdown by certain soil fungi of simple aromatic compounds containing one or more methoxyl groups.

METHODS

Organisms. The following fungi were used: *Haplographium* sp. from deciduous woodland soil; *Hormodendrum* sp. no. 1 from sand under Scots Pine in an afforested sand dune; *Penicillium* sp. no. 13 from moorland soil. These fungi were all used previously (Henderson & Farmer, 1955; Henderson, 1956).

Cultural conditions. Much of the work was based on an adaptation of Kluyver & van Zijp's (1951) technique; 250 ml. conical flasks containing 100 ml. of mineral salts solution (NaNO_3 , 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; KH_2PO_4 , 0.5 g.; KCl, 0.25 g. dissolved in 500 ml. water), to which were added 1 g. glucose and 0.5 g. yeast extract, were inoculated with fungal spores and incubated at 21° for 7 days, by which time a mat of mycelial growth covered the surface of the medium. The medium was poured off and the mat washed three times with sterile distilled water. Sterile solutions of the acids, brought to pH 6.5 before autoclaving, were then added to the flasks. All

operations were carried out aseptically. After further incubation periods the solutions were analysed.

Chromatographic analysis. In this instance 0.1% (w/v) solutions were used beneath the fungal mats. At intervals, usually over a period of about 12 days, a flask was removed from the incubator and the solution was poured off, filtered, acidified and extracted 3 times with 10 ml. ether. The ether was removed and the residue dissolved in ethanol and applied to No. 1 Whatman filter-paper. Each solution was spotted 8 times by means of a capillary pipette. The papers were developed by the descending method using *n*-butanol/ammonia (sp.gr. 0.880)/water (80/5/15 vol.) over a period of 16 hr. at 21°. Two papers were used for each extract, one being sprayed with diazotized sulph-anilic acid (Bray, Thorpe & White, 1950) for detection of phenols, the other with a buffered spray (Fewster & Hall, 1951) for detection of acids.

Spectrochemical analysis. For this purpose 0.01M solutions were used beneath the fungal mats and 1 ml. samples removed aseptically from each flask at intervals of a few days, the frequency of sampling being governed by the rate of disappearance of the compound under investigation. The samples were diluted to 5 ml. and the disappearance of the compounds followed by studying their absorption in the ultraviolet region.

Methoxyl determination. The solution (initially 0.01M) was decanted from the flask and the mat washed 3 times with distilled water. The solution and washings were filtered and evaporated to dryness over a water-bath. The residue was further dried in a vacuum desiccator until of constant weight. The methoxyl determination was then carried out by the method of Vieboch & Brecher (1930). To determine their methoxyl content the mycelial mats were dried in a vacuum desiccator, powdered and weighed. The powder was returned to the desiccator and left until its weight became constant. Methoxyl determination was then carried out as above.

Respiration studies. The spores were incubated overnight in the presence of mineral salts, buffer and yeast extract. These solutions and the conditions and methods were as previously described (Henderson, 1956). Solutions of the substances under investigation were tipped in from the side-arms on the following morning.

RESULTS

Metabolism of monomethoxybenzoic acids by soil fungi

Decomposition of monomethoxybenzoic acids. Mycelial mats of *Hormodendrum* sp. were incubated on 0.01M solutions of the three monomethoxybenzoic acids brought to pH 6.5. Samples were removed from the flasks at intervals, and the disappearance of the acids was followed by the decrease in absorption in acid solution in which the maxima for *o*-, *m*- and *p*-methoxybenzoic acids are at 296, 296 and 257 m μ ., respectively. The results showed that *p*-methoxybenzoic acid was much more rapidly attacked than were the *o* and *m* compounds. After 14 days the concentration of *p*-methoxybenzoic acid was reduced to 9.8% of the original concentration, while 71.5% of *m*-methoxybenzoic acid and 89% of *o*-methoxybenzoic acid remained.

Respiration studies with monomethoxybenzoic acids. At 0.0083M concentrations of the acids (Table 1), *Haplographium* sp. and *Hormodendrum* sp. showed increased oxygen uptakes which were greatest in the presence of the *p* form. In the presence of the *o* and *m* forms the uptakes by *Haplographium* sp. were similar, while *Hormodendrum* sp. gave a marked increase with the *m*

Table 1. *Respiration of Haplographium sp., Hormodendrum sp. and Penicillium sp. on monomethoxybenzoic acids*

1 ml. spore suspension added to vessels (*Haplographium*, 8×10^8 ; *Hormodendrum*, (a) 4.8×10^8 ; (b) 4.7×10^8 ; *Penicillium*, (a) 8.13×10^8 ; (b) 8.36×10^8 ; (c) 8.66×10^8 spores/ml.) + 0.5 ml. mineral salts solution + 0.5 ml. buffer (*Haplographium* and *Hormodendrum* Sørensen's phosphate buffer, pH 5.3; *Penicillium* McIlvaine's citrate + phosphate buffer, pH 3.0) + 0.5 ml. yeast extract (*Haplographium* and *Penicillium* 0.125%; *Hormodendrum* 0.48% (w/v)). Centre cup contained 0.2 ml. 5% (w/v) KOH; 0.5 ml. substrate acid solution (neutralized) tipped in from side-arms.

Organism	Concentration of acid in vessel (M)	Oxygen uptake over the 5 hr. period following the addition of substrates (μ l.)			
		<i>o</i> -Acid	<i>m</i> -Acid	<i>p</i> -Acid	Control
<i>Haplographium</i> sp.	0.0083	177	169	300	18
<i>Hormodendrum</i> sp. (a)	0.0083	52	148	213	40
	(b) 0.0017	42	99	167	41
<i>Penicillium</i> sp. (a)	0.0083	140	82	240	252
	(b) 0.0017	228	169	208	264
	(c) 0.0010	178	176	189	159

form only. When the concentration of the acids was decreased to 0.0017M the oxygen uptakes by *Hormodendrum* sp. in the presence of all three were correspondingly decreased. The small uptake with *o*-methoxybenzoic acid at the higher concentration was therefore indicative of a low ability to attack this acid and was not a result of an inhibitory concentration. The oxygen uptake by *Penicillium* sp. was markedly inhibited by *o*- and *m*-methoxybenzoic acids at 0.0083M, while in the presence of *p*-methoxybenzoic acid at the same concentration it was similar to that of the control. When the concentration of the acids was decreased to give a final concentration in the vessels of 0.0017M, the inhibition by the *o* and *m* acids was decreased. At 0.001M the oxygen uptake in the presence of either acid was greater than that of the control.

Conversion of methoxy to hydroxy groups in monomethoxybenzoic acids. An investigation of the products of decomposition of the monomethoxybenzoic acids was made by paper chromatography. Mycelial mats of *Haplographium* sp., *Hormodendrum* sp. and *Penicillium* sp. were incubated on 0.1% (w/v) solutions of *o*-, *m*- and *p*-methoxybenzoic acids. The solutions were extracted and analysed by paper chromatography at intervals of a few days and in each case the corresponding hydroxy-acid was found in the extract. Only faint traces of *o*- and *p*-hydroxybenzoic acids were formed, but very strong spots of *m*-hydroxybenzoic acid were obtained.

In addition to *o*-hydroxybenzoic acid, *Haplographium* sp. and *Penicillium* sp. produced from *o*-methoxybenzoic acid an unidentified substance which reacted with diazotized sulphanilic acid to give a purple spot of R_F 0.13. Also, from *p*-methoxybenzoic acid, *Penicillium* sp. formed *p*-methoxyphenol.

visible on the papers sprayed with diazotized sulphanilic acid as a lilac spot of R_f 0.94.

Confirmation of the *m*-methoxybenzoic→*m*-hydroxybenzoic acid conversion was obtained by spectrochemical analysis. The extract from a culture in which *Haplographium* sp. had been incubated over *m*-methoxybenzoic acid for 2 days was applied 6 times to each of 5 spots on a chromatography paper. The paper was developed with butanol/ammonia/water, dried, and a strip cut from each side. One strip was sprayed with diazotized sulphanilic acid, the other with buffered indicator spray so that the hydroxy acid and residual methoxy acid could be located. The corresponding portion of the paper containing the hydroxy acid was then cut out and the acid extracted by cutting this portion into smaller strips which were immersed in 5 ml. 0.1% (w/v) NaOH maintained at 50° for 15 min. This solution of the acid was then cooled and examined by u.v. spectrometry. The extract showed the maxima of *m*-hydroxybenzoic acid at 297 m μ . (acid solution) and at 312 m μ . (alkaline).

The formation of *p*-methoxyphenol from *p*-methoxybenzoic acid was also confirmed in a similar manner. The extract, which contained residual *p*-methoxybenzoic acid and *p*-hydroxybenzoic acid in addition to *p*-methoxyphenol, was dissolved in ether and extracted twice with 1% (w/v) sodium bicarbonate solution to remove the acids. The ether layer was then washed once with slightly acidified water and once with water. The ether was removed and the residue used for spectrochemical analysis. Its ultraviolet and infrared spectra were the same as those of authentic *p*-methoxyphenol.

The fate of the methoxyl group on conversion of monomethoxy to monohydroxybenzoic acids. This was studied by estimating the methoxyl content of a solution of *p*-methoxybenzoic acid after 6 days of incubation under a mat of *Hormodendrum* sp. Spectrochemical analysis showed that by this time the concentration of the acid was decreased to 30% of the original concentration. Therefore a comparison of the total residual methoxyl content with the residual methoxy-acid content would indicate whether the methoxyl group was being transferred to another molecule or whether it was being removed entirely from the solution. Chemical analysis in fact showed that the methoxyl content had been decreased to 31.29% of the original concentration, a figure which corresponds closely with the decrease in concentration of the acid.

It was thought possible that the methoxyl groups were being transferred to the mycelium in some way and accumulating there. In order to examine this possibility two mycelial mats of *Hormodendrum* sp., treated in a similar manner except that one was incubated over *p*-methoxybenzoic acid and the other over distilled water for 6 days, were washed, dried and powdered. Estimation of the methoxyl contents of the powders revealed that the different mats had the same methoxyl content, 0.586% for that incubated over the methoxy acid and 0.581% for that incubated over distilled water. These results indicate that if the methoxyl group were being transferred to another molecule, as seems probable, this molecule did not accumulate but was rapidly decomposed. A further possibility is that a volatile compound was being released. Attempts to trap and identify volatile products were not successful.

Metabolism of hydroxybenzoic acids formed from methoxybenzoic acids

Decomposition of monohydroxybenzoic acids. To follow the relative rates of disappearance of the hydroxybenzoic acids, mats of *Hormodendrum* sp. were incubated over 0.01M solutions of the three acids brought to pH 6.5 and samples were removed at intervals for spectrochemical analysis. The disappearance of the acids was followed by the decrease in their absorption in alkaline solution in which the maxima for *o*-, *m*- and *p*-hydroxybenzoic acid are 296, 311 and 280 m μ ., respectively. The results showed that *m*- and *p*-hydroxybenzoic acids were quickly metabolized and after 10 days the latter had almost disappeared while the former was decreased to 7% of the original concentration. Salicylic acid was removed much more slowly, and after 10 days 57% of the original amount was still present.

Table 2. *Respiration of Haplographium sp., Hormodendrum sp. and Penicillium sp. on monohydroxybenzoic acids*

1 ml. of spore suspension added to vessels (*Haplographium*, 5.9×10^8 ; *Hormodendrum*, 8.7×10^8 ; *Penicillium*, 7.96×10^8 spores/ml.) + 0.5 ml. mineral salts solution + 0.5 ml. buffer (*Haplographium* and *Hormodendrum* Sørensen's phosphate buffer, pH 5.3; *Penicillium* McIlvaine's citrate + phosphate buffer, pH 3.0) + 0.5 ml. yeast extract (*Haplographium* and *Penicillium* 0.125%; *Hormodendrum* 0.48% (w/v)). Centre cup contained 0.2 ml. 5% (w/v) KOH. 0.5 ml. substrate acid solution (0.05M, neutralized) tipped in from side-arms to give a final concentration of 0.0083M in vessels.

Organism	Oxygen uptake over the 5 hr. period following the addition of substrates (μ l.)			
	<i>o</i> -Acid	<i>m</i> -Acid	<i>p</i> -Acid	Control
<i>Haplographium</i> sp.	115	68	234	30
<i>Hormodendrum</i> sp.	145	101	166	72
<i>Penicillium</i> sp.	17	329	482	196

Respiration studies with monohydroxybenzoic acids. These acids were all oxidized by the four fungi (Table 2), with the exception of *o*-hydroxybenzoic acid which was inhibitory towards *Penicillium* sp. at the concentration used. The remaining fungi oxidized *o*-hydroxybenzoic acid more rapidly than they oxidized *m*-hydroxybenzoic acid. Of the three acids, *p*-hydroxybenzoic was oxidized most rapidly by all four fungi.

Further conversion of monohydroxybenzoic acids. It is well established for bacteria that *p*-hydroxybenzoic acid is converted to protocatechuic acid (3:4-dihydroxybenzoic acid) before rupture of the ring, with the subsequent formation of β -ketoadipic acid (Evans, 1947; Evans, Parr & Evans, 1949). Walker & Evans (1952) showed that *Pseudomonas fluorescens* grown on *m*-hydroxybenzoic acid was simultaneously adapted to gentisic acid (2:5-dihydroxybenzoic acid). They did not identify the product of ring cleavage. Evans (1947) found that *Vibrio* sp. converted *m*-hydroxybenzoic acid to protocatechuic acid. According to Walker & Evans the pathway of oxidation of salicylic acid by *Ps. fluorescens* is of a different type and organisms grown on this acid were simultaneously adapted to catechol and to the conversion of

cis-cis muconic acid to β -ketoadipic acid. Evans found that *Vibrio* sp. had no action on salicylic acid.

Haplographium sp., *Hormodendrum* sp. and *Penicillium* sp. were incubated on solutions of the acids, and the solutions were examined by paper chromatography as before. From *Hormodendrum* sp. and *Penicillium* sp. cultures on *p*-hydroxybenzoic acid spots with R_F corresponding to that of protocatechuic acid were obtained after incubation periods of 2 and 6 days. The spot from the 2-day *Hormodendrum* sp. culture was extracted from the paper by the method already described for the extraction of *m*-hydroxybenzoic acid. In this instance the solvent used to obtain separation of protocatechuic acid from residual *p*-hydroxybenzoic acid was *n*-propanol/ammonia/water (80/5/15 vol.). The protocatechuic acid was extracted from the papers with borax phosphate buffer (pH 7) and phosphate buffer (pH 6.9). In borax buffer the maxima obtained were at 293 and 253 m μ . (Swain, 1954) and in phosphate buffer at 250 m μ . These are identical with the maxima of authentic protocatechuic acid. There was no trace of protocatechuic acid in *Haplographium* sp. cultures.

Metabolism of di- and tri-methoxybenzoic acids

Respiration studies. These were carried out with 2:4- and 3:4-dimethoxybenzoic acids and 3:4:5-trimethoxybenzoic acid (Table 3). Only *Haplographium* sp. and *Hormodendrum* sp. showed any increase in oxygen consumption. These increases were small compared with those obtained in the presence of the mono-

Table 3. *Respiration of Haplographium* sp., *Hormodendrum* sp. and *Penicillium* sp. on di- and tri-methoxybenzoic acids

1 ml. of spore suspension added to vessels (*Haplographium*, 5.22×10^8 ; *Hormodendrum*, 4.03×10^8 ; *Penicillium*, (a) 6.0×10^8 ; (b) 6.86×10^8 spores/ml.) + 0.5 ml. mineral salts solution + 0.5 ml. buffer (*Haplographium* and *Hormodendrum* Sørensen's phosphate buffer, pH 5.3; *Penicillium* McIlvaine's citrate + phosphate buffer, pH 3.0) + 0.5 ml. yeast extract (*Haplographium* and *Penicillium* 0.125%; *Hormodendrum* 0.48% (w/v)). Centre cup contained 0.2 ml. 5% (w/v) KOH; 0.5 ml. substrate acid solution (neutralized) tipped in from side-arms.

Organism	Concentration of substrate acid in vessels (M)	Oxygen uptake over the 5 hr. period following the addition of substrates (μ l.)			
		2:4-Dimethoxy	3:4-Dimethoxy	3:4:5-Trimethoxy	Control
<i>Haplographium</i> sp.	0.0083	109	94	88	83
<i>Hormodendrum</i> sp.	0.0083	62	59	42	36
<i>Penicillium</i> sp. (a)	0.0083	119	91	97	170
(b)	0.0017	297	290	330	298

methoxybenzoic acids. Thus *Haplographium* sp., which gave marked increases with the *o*-, *m*- and *p*-methoxybenzoic acids, gave a small increased uptake only with 2:4-dimethoxybenzoic acid. Similarly, *Hormodendrum* sp., which showed marked increases with *m*- and *p*-methoxybenzoic acids, gave only small increases with the dimethoxybenzoic acids. *Penicillium* sp. was inhibited by all the acids at 0.0083M, but the inhibition was not obtained when the

concentration of the acids was reduced to 0.0017 M (cf. monomethoxybenzoic acids).

Decomposition of veratric acid. The metabolism of veratric acid (3:4-dimethoxybenzoic acid) by *Penicillium* sp. was followed further by absorption spectrometry. A mat of the fungus was incubated on a 0.01 M solution of the acid brought to pH 6.5. Paper chromatography indicated that vanillic acid was formed from veratric acid by *Penicillium* sp. (see later) and confirmation of this was obtained by absorption spectrometry. The disappearance of the dimethoxy acid was followed by the decrease in absorption at its maximum at 252 m μ . in alkaline solution, while the formation of vanillic acid was traced by the increase in absorption at its maximum at 300 m μ . in alkaline solution. The concentration of veratric acid fell to 79 % of the original concentration after one day and thereafter it fell slowly to 39.5 % of the original after 21 days. Vanillic acid reached its maximum concentration (1.1×10^{-4} M) after 1 day, and thereafter its concentration fell very slowly and was decreased to half of the above concentration after 21 days. Previously evidence was obtained (Henderson, 1956) that vanillic acid was oxidized by an adaptive enzyme, which may explain the initial accumulation of the acid when formed from veratric acid followed by a gradual diminution in concentration after the formation of the necessary enzyme.

Conversion of methoxy to hydroxy groups in di- and tri-methoxybenzoic acids. Conversion of the methoxy group in the *p* position of veratric acid (3:4-dimethoxybenzoic acid) took place in the presence of *Penicillium* sp., a strong spot of vanillic acid (3-methoxy-4-hydroxybenzoic acid) being visible on the paper chromatogram after 3 days. Traces of vanillic acid were also present in *Hormodendrum* sp. cultures but none could be identified in *Haplographium* sp. cultures.

The three fungi *Haplographium* sp., *Hormodendrum* sp. and *Penicillium* sp. each formed from 2:4-dimethoxybenzoic acid a substance which gave an orange-brown spot of R_F 0.38 with diazotized sulphanilic acid. In addition, an orange spot of R_F 0.13 was obtained particularly with *Penicillium* sp. It was not found possible to identify these products by the techniques available.

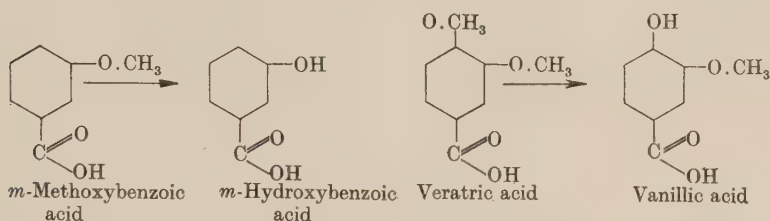
Conclusive results could not be obtained with 3:4:5-trimethoxybenzoic acid. It was expected that conversion of the methoxy group in the *p* position (as with 3:4-dimethoxybenzoic acid) would give rise to syringic acid. Faint traces of this acid (R_F 0.06) were identified from *Haplographium* sp. and *Penicillium* sp. cultures. In addition, a faint orange spot of R_F 0.12 was present in cultures of all four fungi; this compound was not identified.

Metabolism of dihydroxybenzoic acids

A series of respiration experiments was carried out with the six dihydroxybenzoic acids. All three fungi gave small increases in oxygen consumption in the presence of most of the acids. It was, however, not possible to place the acids in any order according to the rates at which they were oxidized.

DISCUSSION

In this study of the de-methoxylation of aromatic compounds by fungi the following conversions have been shown to take place:



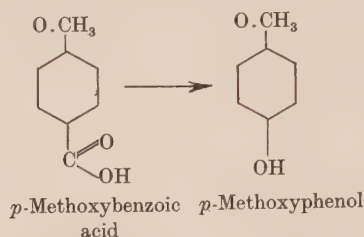
In addition evidence has been obtained by paper chromatography that *o*- and *p*-methoxybenzoic acids are also converted to the corresponding hydroxybenzoic acids. It is possible that de-methoxylation takes place by a transference of the methyl group. Little is known concerning such a process in fungi although a number have been shown to form trimethylarsine from arsenic compounds (Challenger, 1945). Transmethylation systems exist in animal tissues, and methyl groups can be transferred from compounds such as choline and betaine (Challenger, 1945). In the present work analysis of the solution and mycelial mat showed that the total methoxyl content of the solution was being decreased, and that there was no accumulation of methoxyl groups in the mycelial mat. Therefore, if transmethylation were occurring the product formed must have been rapidly metabolized or a volatile compound may have been produced, but attempts to trap any such products were not successful.

The formation of protocatechuic acid from *p*-hydroxybenzoic acid has not been demonstrated before for fungi. However, this step is well established in the metabolism of *p*-hydroxybenzoic acid by bacteria and the present work indicates that the fungi used here metabolize this acid by a similar pathway. It has not yet been possible to identify the products formed on rupture of the benzene ring.

Also of interest is the fact that a methoxybenzoic acid is most rapidly attacked when the methoxyl group occupies the position *para* to the carboxyl group. This was shown by spectrochemical analyses and by respiration studies. Experiments with the hydroxybenzoic acids also showed that the *p* acid was most rapidly metabolized. Furthermore, the conversion of veratric acid to vanillic acid revealed that a methoxyl group in the *p* position is preferentially attacked. These results may be compared with those of Gundersen & Jensen (1956) who, in a study of the decomposition of nitro-phenols, came to the conclusion that *p*-orientation of the groups is important.

Penicillium sp. differed from the other fungi in forming *p*-methoxyphenol from *p*-methoxybenzoic acid, in addition to *p*-hydroxybenzoic acid. There was no evidence of a similar conversion of the other methoxybenzoic acids by this fungus. This reaction is similar to that obtained by Sloane, Crane & Mayer

(1951) when they incubated *Mycobacterium smegmatis* on *p*-aminobenzoic acid and obtained *p*-aminophenol. In this instance the intermediate was aniline.



The ability of the present soil fungi to demethylate compounds in which the methoxyl group is attached to the benzene ring may be an important factor in the transformation of lignified plant materials taking place in soil. One feature of this transformation is the reduction of the methoxyl content which Breger (1951) attributes (for peat) to biochemical removal of the methyl groups from lignin with the production of methane and phenolic hydroxyl groups.

I wish to express my thanks to Dr D. M. Webley for his continued interest and advice. I am very grateful to Dr V. C. Farmer for carrying out the spectrochemical analyses and I also wish to thank Dr R. I. Morrison for the methoxyl determinations and Miss D. Brebner for technical assistance.

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A Virus-inactivating System from Tobacco Leaves

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SUMMARY: Purified preparations of the Rothamsted tobacco necrosis virus made by sedimenting the virus from freshly expressed sap lose infectivity slowly at 0° and rapidly at 18°. Stable infective preparations can be made by ultracentrifugation provided the sap is first frozen or allowed to age; unstable preparations can be stabilized by prolonged centrifugation at 8000 g, or by incubation with citrate and azide. Stable virus preparations lose their infectivity when exposed to the material that sediments from leaf sap centrifuged at 4000-8000 g. This inactivation demands air and is prevented by the presence of azide, but when the sedimented material is kept in air at 0° for some hours a low-molecular weight substance separates from it, and this inactivates the virus whether or not air or azide are present. The material sedimented from the sap of uninfected tobacco leaves, or leaves infected with tobacco mosaic virus, inactivates virus less readily than does material from leaves infected with tobacco necrosis or tobacco ringspot virus. The sediments inactivate tobacco ringspot but not tobacco mosaic virus. The nature of the inactivating substance made by the sediments is unknown, but aldehydes and derivatives of ascorbic acid have comparable effects. Inactivated virus preparations are still serologically active and resemble active ones in all other properties studied.

The ratio of infectivity to the amount of specific antigen contained in purified preparations of the Rothamsted culture of tobacco necrosis virus (RTNV) varies with different preparations and depends on the treatments that are applied to infected leaves or to extracts from them. Although we (Bawden & Pirie, 1950*a*) remarked of these phenomena that 'few are definitely interpretable and many are not consistently reproducible', at that time there did seem substantial evidence that virus particles could lack infectivity when first released from infected leaves and acquire this property *in vitro*. A main reason for this conclusion was that virus preparations made by ultracentrifuging freshly expressed sap at 0°, conditions we thought most likely to yield and maintain the virus in its initial state, were less infective than preparations made by rougher methods or by sedimenting the virus after the sap had stood for some time, and this despite the presence in sap of low molecular weight components that can destroy infectivity (Bawden & Pirie, 1950*a, b*).

Later work made this conclusion increasingly improbable and we have already disclaimed it (Pirie, 1953; Bawden & Pirie, 1956). Our first approach to studying these variations was to compare the infectivities of leaf extracts made in different ways and exposed to different conditions. Studies with more than 100 separate batches of leaves have made results more systematic, but the systems involved are too complex and the effects often too small to be interpreted confidently. These studies showed the fallacy of our assumption that the virus would remain most nearly in its original state when sedimented from the environment of sap as quickly as possible. They showed that partially

purified preparations of RTNV lose infectivity when exposed to conditions that have little or no effect on the infectivity of highly purified preparations or of infective sap. This suggested a direct approach by looking for inactivating systems in the partially purified preparations. Our latest results suggest that, in freshly expressed sap, there is a system which sediments along with the virus and produces substances that destroy infectivity. Ultracentrifugation therefore concentrates both the virus and the inactivating system, and creates conditions in which infectivity is soon affected even at 0°. Either the system itself, or its association with sedimentable particles, is unstable in the conditions obtaining in sap, and when ultracentrifugation is increasingly delayed less of the inactivating system appears in the final virus preparations, which are correspondingly more stable.

This interpretation seems to be confirmed by the fact that we can now make relatively stable preparations of RTNV and then remove their infectivity by exposing them to other preparations made by methods designed to preserve the inactivating system. Work with mixtures of stable, highly purified RTNV and concentrated preparations of the inactivating systems gives unequivocal results, and for the sake of clarity we shall illustrate the phenomenon by describing experiments with such mixtures.

The inactivating system is not specific to tobacco leaves infected with RTNV, and its inactivating action is not confined to RTNV, though it does not extend to all plant viruses. We do not know whether the system has any significance *in vivo*, and for this reason we have not continued our work to the point of identifying the substances that destroy infectivity. Although the system is of questionable biological importance, it is important because of the extent to which ultracentrifugation is now used to make virus preparations, and we publish our results partly to show that misleading results may come from this technique.

METHODS

The virus culture was derived by continuous propagation of the one used earlier, and we have no evidence that it has changed in any way that affects our results. It still produces only necrotic local lesions in the three species of plants we infect, tobacco (*Nicotiana tabacum*, var. White Burley), *N. glutinosa* and French bean (*Phaseolus vulgaris*, var. Prince). Purified preparations still contain specific particles of more than one size, and the pellets sedimented from them by ultracentrifugation still have the characteristic crystalline structure (Bawden & Pirie, 1945). Most of our experiments were made on extracts from infected tobacco leaves, which were treated and inoculated as already described (Bawden & Pirie, 1945, 1950*a*). Inoculum for the batches of tobacco leaves usually came from *N. glutinosa* to decrease the risk of contamination with tobacco mosaic virus. Infectivity assays were made in French bean; these, and precipitation tests with RTNV antisera, were again made by methods already described. Tobacco ringspot virus was propagated in tobacco, and its infectivity was also assayed by counting local lesions produced in tobacco.

Preparation of the virus-inactivating system. Tobacco leaves were picked

when well covered with lesions but before they were wholly shrivelled, usually about a week after inoculation, but the actual time varied between 6 and 12 days depending on the temperature, the infectivity of the inoculum and the susceptibility of the batch of plants. The mid-ribs were cut out, the laminae minced in a chilled mincer, and the sap pressed from the mince by hand through fine cloth into a jar surrounded by ice. The sap was immediately centrifuged for 5 min. at 7000 rev./min. (4000 g) and the supernatant fluid centrifuged again for 20 min. at 10,000 rev./min. (8000 g). Both centrifugations were done in a cold room and the preparations were kept always near 0° because the system is soon destroyed at higher temperatures. The first sediment, which consists mainly of starch, chloroplasts and their fragments, and pieces of cell wall, was discarded, and the second supernatant fluid was kept as the source of virus. The second sediment, which contains the bulk of the virus-inactivating system, was suspended in a volume of water equal to one-fifth the volume of the original sap and immediately centrifuged again at 10,000 rev./min. The second sediment was again suspended in water and its dry matter determined by drying a sample *in vacuo* and weighing.

Preparations made in this way are dark green and contain 0.6–1.0% P, which is mainly in the form of a nucleoprotein that hydrolyses when incubated at 18° for a few hours with salts, especially citrates (Pirie, 1950). For convenience we shall call them preparations of mitochondria, but no anatomical or chemical significance should be attached to the name. Other preparations, differing from the mitochondria in appearance and constitution and, weight for weight, about one-quarter as active in destroying the infectivity of RTNV, can be made by more prolonged centrifugation of the second supernatant fluid at 10,000 rev./min. The resulting sediment contains from 1–2% P and little or no chlorophyll, so there is no reason to relate the system that inactivates RTNV with either the nucleoprotein or the chromoprotein present in the more active preparations.

Preparations of stable infective virus. The simplest way to get stable highly infective preparations of RTNV is to keep the supernatant fluid from the 10,000 rev./min. centrifugation for a day or two at room temperature, because the virus-inactivating system is then rendered ineffective and much of the normal protein denatures. Most of our preparations were made after this preliminary treatment. The stored sap was clarified by centrifugation at 7000 rev./min. and the virus was then either sedimented by centrifugation at 40,000 rev./min. (80,000 g) or concentrated by freezing-out most of the water. The purification was completed by methods already described (Bawden & Pirie, 1950*a*). We sometimes used another method that gave preparations of comparable stability and infectivity. The supernatant fluid from the 10,000 rev./min. centrifugation was centrifuged at 40,000 rev./min. while fresh and the bulky sediment resuspended in water and immediately incubated with M/20 (pH 6) sodium citrate and 2 g./l. neutral sodium azide. This incubation destroys contaminating nucleoprotein and the virus-inactivating system without much affecting the infectivity, and after clarifying the incubated solutions, the virus can be further purified.

Tests for inactivation. For tests of their ability to inactivate RTNV, preparations of the sediments obtained at 10,000 rev./min. are conveniently used at about 10 g./l. At this concentration they remain active for a few days at 0° and a month or so at -20°. Preparations retain their activity at 0° for longer when they contain 2 g. sodium azide/l. Before they were used in experiments, preparations stored with azide were diluted with 50-100 times their volume of water and sedimented at 10,000 rev./min. Inactivation tests were usually made with mixtures containing 10 g./l. of the inactivating system and from 0.2 to 1.0 g. purified virus/l.; the amount of virus was varied according to the infectivity of the preparation and the susceptibility of the test plants. The ratio of 10 or more parts of the mitochondrial preparation to one of virus is greater than would normally occur during the processes of virus purification and was used to ensure clear-cut results. At the end of the incubation period, the mixtures were diluted 1/100 with water, which stops any further inactivation of the virus, and were then inoculated to bean plants at this dilution and at a further dilution of 1/10. No buffer was added, but the pH value of mixtures that inactivated was often measured and was always between 5.5 and 6.5; the virus is stable over a wider range than this.

RESULTS

Properties of the inactivating system

Freshly made preparations of the inactivating system contain some infective virus, which is tenaciously held through repeated sedimentations in the cold, but infectivity is soon lost at 18°. In the experiment recorded in Table 1 a sample of a 10 g./l. preparation made within 2 hr. of harvesting infected

Table 1. *The effect of washing and of incubation on the infectivity of a mitochondrial preparation*

Part of a 10 g./l. mitochondrial preparation from leaves infected with RTNV was incubated at 18°. Another part was diluted to 0.1 g./l., a sample stored at 0°, and the remainder centrifuged at 10,000 rev./min. The sediment was resuspended at 10 g./l., sampled and treated like the original preparation. The second sediment was also resuspended at 10 g./l. and incubated at 18°, and a sample at 0.1 g./l. was stored at 0°. Inoculations were made 20 hr. later with all samples at 0.1 g./l. and the results are given as the numbers of lesions produced on six half leaves.

Preparation	Storage	Infectivity
Original	20 hr. diluted, at 0°	78
	20 hr. undiluted, at 18°	0
Sedimented once	20 hr. diluted, at 0°	54
	20 hr. undiluted, at 18°	0
Sedimented twice	20 hr. diluted, at 0°	47
	20 hr. undiluted, at 18°	0

leaves was incubated at 18°, the rest was diluted 1/100 and most was centrifuged at 10,000 rev./min. while a part was stored at 0°. The sediment was suspended and re-sedimented twice. This repeated washing neither removed

the virus from the sediment nor destroyed the ability of the sediment, when incubated at 18°, to inactivate the virus it contained.

Table 2 shows the rate at which fresh preparations lose their infectivity when kept at 18° and that the loss is prevented or decreased when air is excluded or when the preparations contain sodium azide. Incubation at 18° alters the material so that, instead of sedimenting only when centrifuged at 7000–10,000 rev./min., it flocculates. This flocculation is also partially prevented by azide.

Table 2. *The infectivity of mitochondrial preparations after exposure to various conditions*

One-tenth ml. lots of a 10 g./l. suspension of mitochondria from infected leaves were kept as specified and then diluted to 1/100 and 1/1000 before comparing their infectivities.

Exposure	Nos. of lesions with preparation diluted	
	1/100	1/1000
20 hr. at 0°, diluted 1/100	84	35
3 hr. at 18°, then diluted and 17 hr. at 0°	59	18
10 hr. at 18°, then diluted and 10 hr. at 0°	3	1
20 hr. at 18°, then diluted	0	0
20 hr. at 18° <i>in vacuo</i> , then diluted	43	24
20 hr. at 18° in 2 g./l. sodium azide, then diluted	80	31

Table 3. *Inactivation of purified RTNV by various concentrations of two mitochondrial preparations*

One-hundredth ml. of 10 g./l. purified RTNV was mixed with 0.09 ml. of mitochondrial preparations already diluted to give the stated concentrations. The mixtures were kept in air at 18° for 20 hr. and then diluted 1/100 and 1/1000 before inoculation to bean leaves.

Condition of incubation	Nos. of lesions with virus diluted to	
	10 mg./l.	1 mg./l.
Virus alone	420	235
Virus with preparation A at 1 g./l.	110	69
Virus with preparation A at 3 g./l.	39	20
Virus with preparation A at 10 g./l.	0	0
Virus with preparation B at 3 g./l.	280	111
Virus with preparation B at 10 g./l.	113	48

Tests with artificial mixtures of purified virus and mitochondrial preparations were made in conditions where the purified virus contributed more than nine-tenths of the original infectivity, so that the fraction contributed by the mitochondria was too small to confuse the results even if it were more stable than indicated by the results in Tables 1 and 2. Table 3 illustrates the differing abilities of mitochondrial preparations, from two different batches of leaves, to inactivate the same preparation of RTNV. Preparation A inactivated completely at 10 g./l., and at 1 g./l. inactivated as much as did B at 10 g./l. Preparation A is by no means the most powerful inactivator we have made: some have diminished to one-third the infectivity of 10 times their

weight of purified RTNV during 20 hr. at 18°. Differences of factors of three in infectivity between virus preparations were common during our work in 1950, and the partially purified virus preparations we were then making might well have contained more than one-tenth of their weight as mitochondrial contaminants. At the various stages when easily sedimentable material was separated from the virus, preparations were centrifuged only briefly at 7000 rev./min. The experiment set out in Table 4 shows that this was not enough to stabilize virus prepared by ultracentrifugation. The starting material for both preparations was fresh sap clarified by centrifugation for 5 min. at 6000 rev./min., and the virus was sedimented twice. The final pellets were stirred with water to give approximately 2 g./l. suspensions. Half of each suspension was then centrifuged for 5 min. at 8000 rev./min., when the supernatant fluid was poured off; the other halves were centrifuged for 12 min. at 10,000 rev./min. and the supernatant fluid carefully siphoned off without disturbing the sediment. The infectivity of both virus preparations, particularly at 18°, was stabilized by the greater centrifugation.

Table 4. *Increase in stability of RTNV brought about by centrifugation*

Preparation 1 was made from leaves five days after inoculation and preparation 2 four days later. Each preparation was centrifuged as specified and samples of the supernatant fluids were kept at 0° and 18° before diluting and comparing their infectivities.

Centrifugation	Storage	Nos. of lesions			
		Preparation 1		Preparation 2	
		1/100	1/1000	1/100	1/1000
5 min. at 8,000 rev./min.	20 hr. at 0°	182	25	221	37
	20 hr. at 18°	15	0	2	0
12 min. at 10,000 rev./min.	20 hr. at 0°	190	30	410	57
	20 hr. at 18°	91	17	102	15

Virus preparations that are more stable than any in Table 4 can be made by repeating the ultracentrifugation and prolonging the clarification at 10,000 rev./min., but it is easier to get stable preparations by methods already described for removing the inactivating system. When centrifugation alone is used, then the mitochondrial fraction needs to be removed by prolonged centrifugation at 10,000 rev./min. at each stage. It is not enough to do this treatment at the end of the ultracentrifugation cycle, unless this is done at 0° and with the minimum of delay, because, as will be shown later, virus-inactivating substances that are not sedimentable are produced as long as mitochondria are present.

Ultracentrifugation encourages the inactivation of RTNV by increasing the concentration of both mitochondria and virus, for concentration is more important than the ratio between the two. A mixture of RTNV and mitochondria that loses all its infectivity in 20 hr. at 18° when incubated at 5 g./l., may lose only half or less when incubated at 1 g./l. and suffer no detectable loss when incubated at 0.1 g./l.

Table 5 compares the relative abilities of five different types of material from tobacco to inactivate RTNV. Three of these were made as already described for mitochondrial preparations, one from uninfected leaves, and two from infected plants, one with RTNV and the other with tobacco ringspot virus. The other two materials were both from leaves infected with RTNV; one was the sediment obtained by centrifuging sap at 7000 rev./min. and the other came from the leaf residue remaining after the leaves were minced and sap extracted. The residue was extracted with water in a high-speed blender and then the extract was centrifuged at 10,000 rev./min. to obtain the sedimentable material. Mitochondrial preparations from plants infected with RTNV

Table 5. *The inactivation of RTNV and tobacco ringspot virus by mitochondrial preparations from different sources*

One-tenth ml. lots of water containing 1 mg. of the specified sediments and 20 μ g. of purified RTNV were exposed to air at 18° for 20 hr. Each was diluted to 4 ml. and tested at this and at a further dilution of 1/10. A partially purified preparation of tobacco ringspot virus was similarly incubated with only 2 of the sediments and diluted 1/100 before testing.

Virus incubated with	Nos. of lesions with		
	RTNV at		Ringspot virus at
	5 mg./l.	0.5 mg./l.	1/100
Water	475	365	250
7000 rev./min. sediment from sap of leaves with RTNV	35	8	—
10,000 rev./min. sediment from sap of leaves with RTNV	3	0	0
10,000 rev./min. sediment from blender extract of leaf residues	360	111	—
10,000 rev./min. sediment from sap of leaves with tobacco ringspot virus	10	0	0
10,000 rev./min. sediment from sap of uninfected tobacco leaves	405	257	—

and tobacco ringspot virus were equally active, but the other preparations were much less so. Table 5 also shows that the mitochondrial preparations from plants infected with RTNV and tobacco ringspot virus inactivated tobacco ringspot virus as readily as RTNV. The activity of mitochondrial preparations from uninfected plants varied and some were more active than the one used for the experiment recorded in Table 5, but we have never made one that was as active as the least-active preparation from leaves infected with RTNV. Tobacco plants infected with tobacco mosaic virus give preparations no more active than those from uninfected plants. French bean leaves infected with RTNV also yield active preparations.

There is no evidence to show whether infection with RTNV or tobacco ringspot virus increases the amount of inactivator or makes it more readily sedimentable at 10,000 rev./min. Both these viruses cause necrotic lesions and the inactivator may increase in dying cells, but it also occurs in considerable amounts in leaves that are infected with tobacco ringspot virus and showing mainly chlorotic lesions.

Preparations from infected and uninfected leaves look alike and have

similar compositions. A few measurements of their O_2 uptake made in Warburg manometers, and of their catalase contents, suggest that infection enhances O_2 uptake and diminishes catalase, but different batches of healthy leaves differ from one another so much that many more batches of plants would need to be studied before this could be stated confidently.

Some other variations in the preparative technique have had no consistent effect on the activity of the preparation. Thus tobacco leaves infected with RTNV were moistened with sucrose solution before they were minced so that the final extract contained 0.5 M sucrose. This procedure stabilizes chloroplasts and other intracellular structures but, in many tests, has never affected the yield or the activity of the virus-inactivating system. Various other modifications to the method of extracting sap have also had little or no effect; these include pressing through a narrow slot (Pirie, 1956) and grinding in a mortar, either alone or with extracting fluids containing neutral ascorbate, cysteine, azide, cyanide, or buffers to raise the pH value to 7.5. By contrast with the lack of effect of these extracting methods, the subsequent treatment of extracts has a great effect. The technique we have described for making infective stable preparations of RTNV shows that freezing the sap and ageing it *in vitro* prevent inactivation. So also does shaking for 1–2 min. with chloroform, acidifying to pH 4.8, or dialysis. All these treatments greatly diminish the amount of material that sediments at 10,000 rev./min. from sap that has already been centrifuged at 7000 rev./min., but the small amount that does sediment is, weight for weight, less active than preparations as normally made. We have no evidence to show whether these treatments destroy the inactivating system, make it sedimentable at 7000 or unsedimentable at 10,000 rev./min. It is worth noting that Steere (1956) found shaking with $CHCl_3$ a useful procedure in purifying tobacco ringspot virus, which is inactivated by the mitochondrial preparation.

Whether or not the inactivating system is destroyed rapidly in sap at 18°, there are still ample explanations for the greater stability of RTNV in sap than in partially purified preparations made by ultracentrifugation. First, the absolute concentration of the inactivator is much smaller and, secondly, sap contains substances that inhibit its action. The second is shown by the fact that mixtures of purified virus and mitochondrial preparations, which soon become non-infective when incubated alone, remain infective when incubated in the presence of either a filtrate from boiled sap or the supernatant fluid from ultracentrifuged sap.

The dissociation of the inactivating system

Table 1 showed that the ability to inactivate RTNV was not readily removed from fresh preparations of mitochondria by washing them in large volumes of water. Such an experiment, however, shows only that the washed sediments were still active and not whether any inactivating substances had been removed from them. Table 6 shows that extracts of the sediments do contain inactivating substances and in amounts that are determined by the intervals between the successive centrifugations. In this experiment a

10 g./l. preparation was sedimented twice from water within one hour of being made. It was re-suspended, left at 0° for 20 hr. and then sedimented again, when it was immediately re-suspended and re-sedimented. The suspension was then left for another 2 days at 0°, when it was again sedimented.

Table 6. *Inactivation of RTNV by successive extracts of a mitochondrial preparation*

A 10 g./l. mitochondrial preparation was sedimented 5 times at the intervals specified, and the sediment was each time re-suspended in the original volume of water. Sediments and extracts were kept at 0° continuously and finally 0.1 ml. lots of the extracts were incubated with 20 µg. of purified virus.

Virus incubated for 20 hr. at 18° with	Nos. of lesions with virus at	
	5 mg./l.	0.5 mg./l.
Water	500	210
1st immediate extract of new preparation	380	137
2nd immediate extract of new preparation	500	162
3rd extract made after lying 20 hr.	115	28
4th extract made immediately after 3rd	260	86
5th extract made after lying 48 hr.	380	165

Finally the five clear and colourless extracts were tested for their ability to inactivate purified RTNV; the third extract obviously contained much inactivator which was not previously extractable. Most of the inactivating substance was released from the sediment during the first day, but this does not always happen. Equally active extracts have been obtained by sedimenting some preparations on 2 or 3 successive days.

When 24 hr. extracts of sediments are no longer able to inactivate RTNV, the sediments themselves also have little inactivating power (Table 7). The material called 'thrice extracted sediment' in Table 7 is the sediment from the third extract used for the experiment in Table 6, and the '9 times extracted sediment' was given four more extractions after being used to provide the fifth extract for the experiment in Table 6. Table 7 also shows that the inactivating power of a sediment was decreased by the presence of CHCl_3 and that the activity of an extract was not destroyed by drying *in vacuo*.

Table 7. *The inactivating power of extracted sediments and dried extract*

Twenty µg. purified RTNV were incubated in 0.1 ml. containing the specified materials and then diluted for testing.

Virus incubated for 20 hr. at 18° with	Nos. of lesions with virus at	
	5 mg./l.	0.5 mg./l.
Water	550	140
1 mg. of thrice extracted sediment	2	0
0.2 mg. of thrice extracted sediment	17	5
1 mg. of thrice extracted sediment with CHCl_3	230	60
1 mg. of 9 times extracted sediment	530	145
0.1 ml. of dried and redissolved extract	60	14

Extracts with virus-inactivating ability can be obtained by extracting sediments at 18°, but less consistently than at 0°. This does not mean that the responsible substance (or substances) is unstable at 18°, for extracts made at 0° remain active when kept at 18°; they also remain active after boiling briefly and after freezing and thawing. When sediments are enclosed in dialysis tubes and kept at 0°, the virus inactivator occurs in the water surrounding the tubes. Unlike the inactivation by sediments, that by extracts or dialysates occurs when air is excluded or azide is present.

A day or so at 18°, or boiling briefly, destroys the ability of sediments to inactivate RTNV. So also does incubation with CHCl_3 , which removes the green colour and within a few hours produces a grey-brown coagulum. Thymol seems not to affect the sediment; as virus is also inactivated in its presence, it provides a suitable disinfectant and experiments with it exclude the possibility that the inactivation is caused by bacteria or other micro-organisms that grow during the incubation at 18°. Azide, which is also a powerful disinfectant, prevents the sediments from inactivating RTNV but does not destroy the inactivating system, and when the azide is removed the sediments regain their activity. The inhibitory effect of excluding air is also reversible. To identify treatments that specifically affect inactivation by sediments, preparations must be freshly sedimented. If they have lain at 0° for some hours or days since the last sedimentation, results are equivocal, because the preparations now combine the features of sediments and extracts.

An obvious explanation of the differences between inactivations by mitochondrial preparations and by extracts from them is that the former contain an enzyme system, which makes a substance that diffuses away and inactivates the virus. The system in the mitochondria seems to be readily destroyed and not to function without air or in the presence of azide. The substance is more stable than the system that makes it, and it can inactivate RTNV whether or not air or azide is present.

Active extracts may contain as little as 100 mg. dry matter/l., and the main components are breakdown products of nucleic acid, for mitochondrial preparations from all leaves contain both nucleoprotein and ribonuclease, and those from leaves infected with RTNV, like preparations of the virus itself when made by ultracentrifugation, are particularly rich in this enzyme (Pirie, 1957). The enzyme and the nucleoprotein interact slowly, but this process is unlikely to be responsible for producing the virus inactivator, because their interaction is not inhibited by azide (Pirie, 1950) and sediments richest in nucleoprotein are not the most effective inactivators of RTNV.

Analogous inactivations

As the inactivation of RTNV by extracts from sediments and by citrate (Bawden & Pirie, 1950*b*) are similar in some ways and both occur when air is excluded or when azide is present, we tested some other substances that can make complexes with metals. At concentrations around 0.01M, oxalate did not inactivate, but ethylenediamine tetraacetate at 0.01 and 0.002M did after some hours at 18°. Hence there is no need to look further for an explanation

of the inactivation than the sequestration from the virus of some metal that is essential for infectivity and that dissociates only slowly. However, there are other agents, active at biologically significant concentrations, which also inactivate RTNV without denaturing it and whose action is not easily interpreted by the sequestration of some essential metal. Aldehydes, such as formaldehyde and phenylacetaldehyde, at 0.003M inactivate within a few hours at 18°. Table 8 shows that phenylglyoxal has some effect even at 0.0001M and that the effect is not on the host, for adding the substance to inocula immediately before they were rubbed over the leaves did not decrease the numbers of lesions.

Table 8. *The inactivation of RTNV by phenylglyoxal*

Samples 1-4 were incubated for 20 hr. in air at 18°; each contained 20 μ g. purified RTNV in 0.1 ml. and was diluted to 4 ml. and tested at this dilution and after further dilution of 1/10. Sample 5 was made like sample 2 but diluted almost immediately, and diluted phenylglyoxal was added to the virus in sample 6.

Treatment of virus	Nos. of lesions after diluting virus to	
	5 mg./l.	0.5 mg./l.
1. Incubated with water	340	100
2. Incubated with 3mm-phenylglyoxal	21	7
3. Incubated with 1mm-phenylglyoxal	150	41
4. Incubated with 0.1mm-phenylglyoxal	170	78
5. Exposed momentarily to 3mm-phenylglyoxal and then diluted	250	91
6. Diluted phenylglyoxal added immediately before testing	380	99

Before we realized that the formation of the virus inactivator from the mitochondrial preparations, and not the final reaction between the inactivator and the virus, was the oxidative process, we made many tests of the effect of oxidizing and reducing systems on the infectivity of RTNV. Although these may not be relevant to inactivation by the extracts of mitochondria, the results with ascorbic acid are interesting enough to warrant recording. We have distinguished three distinct effects. Virus preparations made from leaves that were infiltrated with sodium ascorbate, or from sap to which ascorbate was added at various stages during the processes of isolating the virus, were only one-quarter to one-half as infective as preparations made without this addition. In these experiments, enough 0.1M neutralized ascorbic acid was added to sap to increase the concentration to 1.0-1.5 g./l., which is 3-4 times the normal level in tobacco sap. By contrast, ascorbate at these concentrations prevents the inactivation of RTNV contained in mitochondria when these are incubated in the conditions used in the experiments set out in Tables 1 and 2. Cysteine and glutathione also prevent this type of inactivation. After 20 hr. at 18° ascorbic acid or —SH is still present and the failure of inactivation is presumably a consequence of the maintenance of reducing conditions.

The third effect is the most interesting. Stable preparations of RTNV lose their infectivity when exposed to ascorbate for a day at 18°, and the inacti-

vation is enhanced instead of inhibited by azide (Table 9). The decreased infectivity produced by exposing the virus briefly to an ascorbate+azide mixture, which had already stood for 20 hr., suggests that the responsible substance inactivates the virus rapidly. The substance itself, however, is produced only slowly in the ascorbate+azide mixture, for in tests when the three components were mixed briefly before being diluted and inoculated, the mixtures produced as many lesions as did the control virus solutions. Less than 10% of the ascorbic acid, as indicated by titration with dichlorophenol-indophenol, disappears in 20 hr. at 18°. The purified RTNV preparations contain little or no ascorbic oxidase and their inactivation by the ascorbate+azide mixture is lessened by excluding air.

Table 9. *The inactivation of RTNV by ascorbic acid and azide*

Ascorbate was used at 0.005M, azide at 0.01M and for each treatment 20 µg. purified RTNV was incubated in 0.1 ml. and then was diluted for testing after the stated time.

Virus at 18° with	Nos. of lesions with virus at	
	5 mg./l.	0.5 mg./l.
Water	650	77
Ascorbate for 20 hr.	124	10
Ascorbate for 2 hr.	230	23
Ascorbate and azide for 20 hr.	4	0
Ascorbate and azide for 2 hr.	350	11
Virus added immediately before testing to a mixture of ascorbate and azide made 20 hr. earlier	370	6

Lojkin (1937) concluded that a peroxide, made during the catalytic oxidation of ascorbate by a metal, was responsible for the inactivation of tobacco mosaic virus by ascorbic acid, and similar interpretations have been advanced for other oxidations brought about by ascorbic acid (Warren, 1943). If this is the mechanism by which RTNV is inactivated, a peroxide other than H_2O_2 is probably responsible, because exposure for 20 hr. at 18° to 0.003M- H_2O_2 affects the infectivity of RTNV only slightly and 0.01M is needed for inactivations comparable to those with ascorbate. There are precedents for azide playing a part in peroxide-induced actions (Theorell & Ehrenberg, 1952; Keilin & Hartree, 1954), but we have no evidence that such actions are concerned in the inactivation of RTNV.

If any form of oxidation is concerned, it is not easily reversed and, although exposure to reducing agents such as ascorbic acid, cysteine and glutathione stops the inactivation of RTNV by mitochondria, these substances do not restore infectivity to preparations that have lost it. Also, repeated sedimentation of the virus does not restore infectivity after any of the inactivation we have discussed.

Similarities between infective and non-infective virus preparations

The inactivation by mitochondrial preparations is not accompanied by any gross changes in the serological character or physical state of the virus preparations. Preparations made non-infective still react with virus antiserum

and give the same precipitin titre and optimal precipitation as when they were infective. Nor does electron microscopy suggest any great change in the particles. Preparations of RTNV contain particles of at least two sizes; the larger particles are more abundant in fresh preparations and the smaller in preparations carried through to the state in which they are less infective and crystallize readily (Bawden & Pirie, 1945, 1950*a*). An obvious explanation would be that the smaller particles are non-infective derivatives of the larger ones. That may explain some types of inactivation. But the inactivations studied here seem not to change large to small particles. The resolving power of our electron microscope is not good enough to identify the particles so precisely that each type can be counted accurately, but preparations made non-infective by sediments, or by extracts from them, still contain large particles and there is nothing to suggest that their proportion has been decreased by inactivation.

The fact that no obvious change accompanies inactivation has two equally plausible explanations. Loss of infectivity may mean a change in only a small part of each virus particle, a change beyond the sensitivity of the methods used. From kinetic studies of the inactivation of tobacco mosaic virus by formaldehyde, Cartwright, Ritchie & Lauffer (1956) concluded that inactivation could result from the reaction of one formaldehyde molecule at one site on a virus particle, perhaps with a hydroxy group on ribose. Such a reaction would not be expected to affect either morphology or serological activity. Alternatively, most of the particles in even the most active preparations may already be non-infective, so that changes in affected particles could be considerable but would occur in too small a fraction of the whole to be detected.

DISCUSSION

Our results adequately explain the variations in infectivity between preparations of RTNV made from sap by different methods, and this point calls for no more discussion. There are, though, other phenomena less readily explained. When the virus is extracted from the residue that remains after leaves are minced and their sap expressed, it is, weight for weight, less infective than virus got from the sap (Bawden & Pirie, 1950*a*). We have confirmed this with many different batches of leaves, and have now further found that the infectivity is greater when supernatant fluid from ultracentrifuged sap is used to extract the virus from the leaf residue than when water is used. These different extracting fluids could act by influencing the extent to which the virus inactivator operates during the course of extraction. While sap is present it seems to be inactive, but when the sap is removed it may come into operation. This simple explanation is not fully adequate, however, because extracts made with sodium azide solution, which prevents mitochondrial inactivation of RTNV *in vitro*, yield virus that is no more infective than virus extracted with water.

Another phenomenon for which there is no adequate explanation is the low infectivity of virus made from the sap of leaves frozen before being minced.

In 1950 we explained this as a failure of an activating mechanism which we then postulated. RTNV does not lose infectivity when minced leaves or sap are frozen and there is not always a loss when intact leaves are frozen. It is more evident when leaves that have been infected for many days and are very chlorotic are frozen at -5 to -10° , than when young leaves are used, or when leaves are frozen quickly at -20° . Neither the inactivation that follows freezing nor that in residues of minced leaves need be correlated with the inactivating system we have described, but they could result from changes in the microanatomy of the leaf that alter the relative disposition of virus particles and the mitochondria.

As extracts from plants infected by viruses are studied in greater detail it becomes increasingly evident that they contain a range of anomalous particles not all of which are infective. Some lack nucleic acid; but some that contain nucleic acid are also not infective. It does not seem that the virus-inactivating system we have described plays any part in producing the non-infective nucleoprotein that is serologically related to tobacco mosaic virus (Bawden & Pirie, 1945, 1956), for when preparations of this virus were incubated with mitochondrial sediments their infectivity was unimpaired. However, with RTNV, tobacco ringspot and other viruses that lose their infectivity more readily than does tobacco mosaic, it could play a part in producing such components, particularly in preparations made by ultracentrifugation. There is at the moment no way of telling whether the system operates at all *in vivo*, though the assumption that it does provides a ready explanation for the fact that preparations of RTNV made from different lots of leaves by a method designed to preserve infectivity *in vitro* can still differ considerably in their relative infectivities. There seems, too, a suggestion of biological significance in the fact that the inactivating system is more evident in leaves infected with viruses affected by the system than in healthy leaves or those infected with tobacco mosaic virus, but work with many more viruses will be needed before this becomes more than a suggestion.

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Pyruvate Accumulation and Development of Thiamine Deficiency in Cultures of *Proteus vulgaris*

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SUMMARY: The accumulation of pyruvate in cultures of *Proteus vulgaris* grown with limiting concentrations of nicotinic acid has been investigated. Nicotinic acid-deficient organisms remaining in contact with a lactate medium under conditions where pyruvate accumulation is occurring gradually become deficient in thiamine. The thiamine content of organisms at various stages of growth was estimated, and a relationship between thiamine content and length of lag phase found. Maximum rates of pyruvate oxidation were obtained in organisms which were not deficient in either nicotinic acid or thiamine. The possible metabolic significance of these findings is discussed.

As shown by Jackson & Copping (1952) the growth curve of *Proteus vulgaris* in a defined medium, with lactate as sole carbon source and limiting concentrations of nicotinic acid, has a characteristic form, the phase of exponential synthesis being followed by a phase of arithmetic linear growth. A preliminary account of pyruvate accumulation in the medium and of the development of thiamine deficiency in the organisms was given by Jackson (1952*a, b*); the present paper presents a detailed account of some of these observations and of additional findings.

METHODS

The organism, medium, conditions of incubation, opacity measurements, dry-weight determinations and measurements of oxygen uptake were as described by Jackson & Copping (1952). Thiamine, in freshly prepared aqueous solution, was added to the medium as required.

Thiamine assays. Organisms from suitable culture samples (40 or 80 ml. according to the concentration of the culture) were collected by centrifugation at 12,500 rev./min. and washed twice with glass-distilled water. They were re-suspended in 5 ml. 0.05 M-acetate buffer (pH 4.5) and were killed by heating in a boiling-water bath for 20 min. Digestion with a papain and taka-diastase mixture and microbiological assay with *Lactobacillus fermentum* were as described by Barton-Wright (1946). This method was used for estimation of the thiamine content of the supernatant fluids of centrifuged samples.

The *pyruvate content* of the medium was estimated by the method of Lu (1939). In early experiments the results obtained by Lu's method were checked manometrically, using the technique described by Umbreit, Burris &

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Stauffer (1949), and no significant difference between the two methods was found. Values are expressed as pyruvic acid.

In experiments on the lag phase of cultures, the following substances were used: β -alanine, *p*-aminobenzoic acid, folic acid, pyridoxin, uracil, coenzyme I, xanthine, riboflavin, calcium-D-pantothenate, 4-methyl-5-hydroxyethylthiazole and 2-methyl 4-amino-5-ethoxymethylpyrimidine. Fresh solutions were prepared and used as required.

Chromatography. In experiments on the accumulation of pyruvate, both in the lag phase and during the late stages of growth, the keto-acid was identified chromatographically; the method of Altmann, Crook & Datta (1951) was used. Pyruvate was the only substance detected.

Coenzyme I. A preparation of coenzyme was made from baker's yeast by the method of Page (in Umbreit *et al.* 1949).

Coccarboxylase. Coccarboxylase was obtained from Roche Products Ltd., Welwyn Garden City, Herts, who also kindly provided the samples of 4-methyl-5-hydroxyethylthiazole and 2-methyl 4-amino-5-ethoxymethylpyrimidine.

Nicotinic acid concentrations. In the medium used, concentrations of 0.02, 0.03 or 0.04 $\mu\text{g.}$ nicotinic acid/ml. were growth limiting, and on the linear portion of a dose-response curve. Concentrations of nicotinic acid of about 0.2 $\mu\text{g.}/\text{ml.}$ were just in excess of maximum requirements at the lactate concentration used. In experiments where the nicotinic acid concentration is described as 'an excess', the concentration of nicotinic acid was 2.4 $\mu\text{g.}/\text{ml.}$

RESULTS

Pyruvate accumulation in the medium

During the phase of exponential growth which occurred before the nicotinic acid concentration became limiting, pyruvate concentrations of 10–15 $\mu\text{g.}/\text{ml.}$ were regularly found in the medium, but at the onset of linear growth the pyruvate concentration began to rise, and this accumulation continued during the later growth period and after growth had ceased (Fig. 1). During the linear growth phase, the rate of pyruvate accumulation increased with time, but when growth had ceased, the rate of pyruvate accumulation remained constant. The effect of adding nicotinic acid to a culture in the linear growth phase is shown in Fig. 2. There was a prompt decrease in pyruvate concentration and restoration of exponential growth. When thiamine or coccarboxylase to final concentration $1.6 \times 10^{-4}\text{M}$ and not nicotinic acid, was added during the phase of linear growth, further accumulation of pyruvate was not prevented, and there was no measurable change in growth rate. The effect of coenzyme I was similar to that of nicotinic acid.

The cultures used were fully grown after about 30 hr. of incubation, and by the time they were 48 hr. old they had accumulated more than 3 mg. pyruvate/ml. (expressed as pyruvic acid). A 48 hr. culture of this kind was divided into five parts and the effects of adding nicotinic acid, thiamine, coenzyme I and coccarboxylase in final concentrations of $2 \times 10^{-5}\text{M}$ were

observed. Nicotinic acid and coenzyme 1 both promoted growth associated with a decrease in pyruvate concentration; thiamine and cocarboxylase brought about a definite but much less rapid decrease, and cocarboxylase was about twice as effective as thiamine on a molar basis.

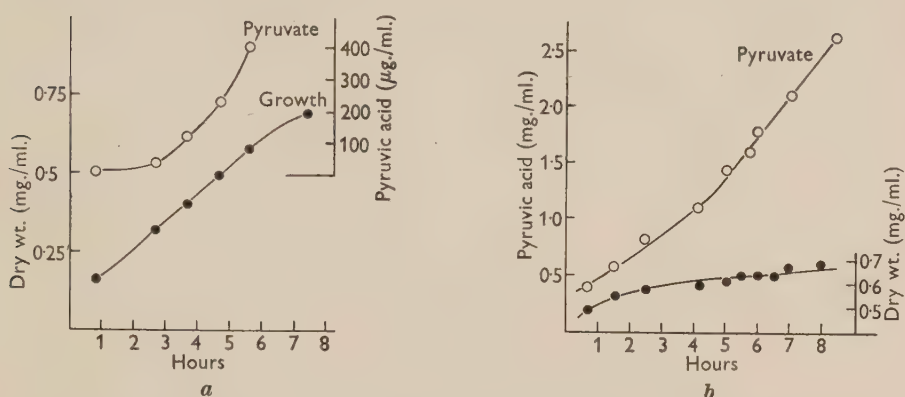


Fig. 1. (a) The early stages of pyruvate accumulation during the linear growth phase. (b) Continuation of pyruvate accumulation after growth had practically ceased.

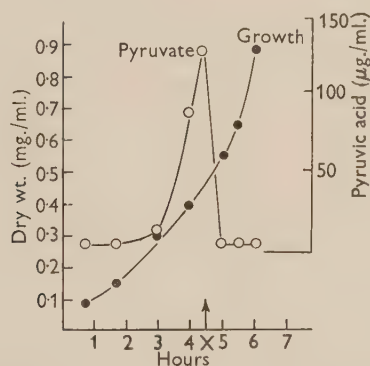


Fig. 2. Effects of adding more nicotinic acid to a culture in the linear growth phase. Nicotinic acid to a final concentration of 2.4 μ g./ml. was added at X.

As would be expected, when growth was limited by lactate concentration and not by nicotinic acid concentration, no pyruvate accumulation was found. When nicotinic acid was limiting, the pyruvate concentrations found at 72-hr. increased as the amount of lactate in excess of growth requirements was increased. In a series of cultures with lactate concentrations of from 1 to 8 mg./ml., and all containing 0.04 μ g. nicotinic acid/ml., no pyruvate accumulation was found in cultures with 1, 2, 3 or 4 mg. lactate/ml., and in these lactate was limiting. When lactate concentrations in excess of 4 mg./ml. were used, pyruvate accumulation occurred, and the concentrations found were roughly proportional to the excess of lactate.

Lag phase phenomena

For investigation of the lag phase of the organisms on transference to fresh medium, organisms were harvested at different times during and after growth and were washed twice with glass-distilled water. They were then re-suspended

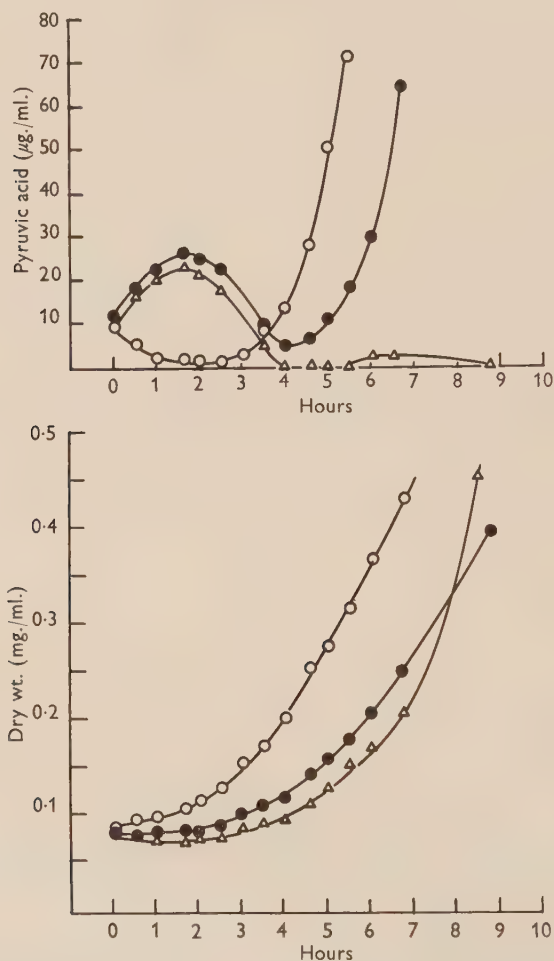


Fig. 3. Curves of growth and pyruvate concentrations in cultures inoculated at time 0 with thiamine-deficient organisms. Three cultures were used, and are represented respectively by circles, dots and triangles in both pyruvate and growth curves. ●—●, 0.03 $\mu\text{g.}$ nicotinic acid/ml.; ○—○, 0.03 $\mu\text{g.}$ nicotinic acid/ml. + 10^{-5}M -thiamine; △—△, 2.4 $\mu\text{g.}$ nicotinic acid/ml.

in fresh medium to a concentration equivalent to *c.* 0.03 mg. dry wt. organisms/ml., and samples were taken at intervals for opacity and pyruvate estimations.

The addition of thiamine+nicotinic acid to the medium did not affect the lag phase of organisms harvested during the linear phase of growth or at

about the time of cessation of growth. With organisms collected at these times the lag phase was short (less than 30 min.).

With organisms collected 8–12 hr. after the cessation of growth, however, the findings were different; they are shown in Fig. 3. The addition of thiamine to final concentration 10^{-5} M strikingly shortened the lag phase, and sometimes practically abolished it. Pyruvate estimations during the lag phase showed that, in cultures without added thiamine, an initial increase of pyruvate concentration of about 25 μ g./ml. was followed by a decrease. Growth in the cultures began when the pyruvate concentration had decreased to *c.* 10 μ g./ml. The addition of an excess of nicotinic acid during the lag phase did not prevent this pyruvate accumulation, and of numerous substances tested only thiamine and cocarboxylase were effective in preventing pyruvate accumulation and in shortening lag. The ineffective factors tested were β -alanine, *p*-aminobenzoic acid, folic acid, pyridoxin, uracil, xanthine, riboflavine and calcium-D-pantothenate. Pyruvate accumulating during the lag phase was identified chromatographically.

Concentrations of added thiamine ranging from 2×10^{-5} M to 2×10^{-8} M had approximately similar effects on the lag phase, with a starting inoculum equivalent to 150 μ g. dry wt. organisms/ml. Two possible precursors of thiamine, 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-ethoxymethylpyrimidine were tested separately and in combination for effects on the lag phase, but were found to be ineffective.

When cultures to which thiamine had been added during the lag phase were followed during their total period of growth, pyruvate was found to accumulate during the linear phase, when the nicotinic acid supply was growth-limiting, as in cultures without added thiamine. The mean generation time in cultures to which thiamine was added initially was rather less, during the exponential phase, than in cultures which had been supplied with nicotinic acid alone, and growth during the linear phase was also slightly faster (Fig. 3). These effects on growth rate were only seen when thiamine was added at the time of inoculating the cultures, and not when it was added after nicotinic acid had become limiting.

Effects of thiamine on O₂ uptake

The alterations in growth and pyruvate accumulation described were accompanied by corresponding effects on oxygen uptake of suspensions of washed organisms. The addition of thiamine to washed suspensions of organisms collected during the linear growth phase or shortly after cessation of growth did not increase the rate of O₂ uptake with pyruvate as substrate and excess of added nicotinic acid. The rate of oxygen uptake of organisms collected 8–12 hr. after the cessation of growth was, however, strikingly greater when thiamine + nicotinic acid were added than when only nicotinic acid was added. Cocarboxylase was slightly more active than thiamine, on a molar basis (Fig. 4).

The thiamine content of organisms

The thiamine content of organisms collected at different times during growth in media containing either limiting concentrations or an excess of nicotinic acid was estimated. Organisms growing exponentially in medium with optimal nicotinic acid concentrations were found to contain 40–50 $\mu\text{g.}$ thiamine/g. dry wt. organisms. Under these conditions, i.e. with excess of nicotinic acid and limiting lactate concentration, this concentration of thiamine

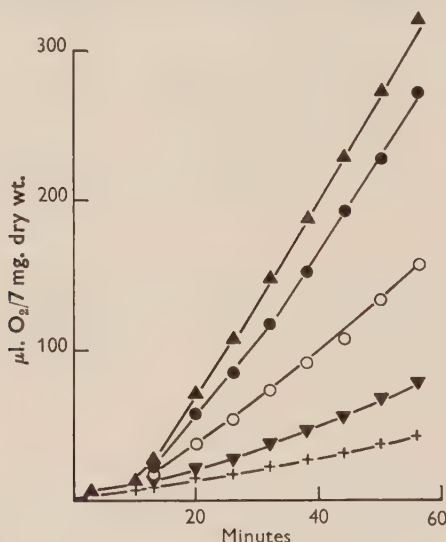


Fig. 4. Oxygen uptake, with pyruvate as substrate, of thiamine-deficient organisms with added nicotinic acid, thiamine and cocarboxylase. +—+, no additions; ▼—▼, cocarboxylase; ○—○, nicotinic acid; ●—●, nicotinic acid + thiamine; ▲—▲, nicotinic acid + cocarboxylase. Final volume in flasks was 3.0 ml. Pyruvate 0.036M; concentration of added factors, $8.3 \times 10^{-6}\text{M}$; MgSO_4 , $2 \times 10^{-4}\text{M}$ present in each flask; phosphate 0.03M, pH 7.4. The thiamine or cocarboxylase was placed in the main compartment of the flasks. Nicotinic acid was added from the side-arm 5 min. after the zero reading. The centre well contained a folded 2 cm.² of filter paper and 0.1 ml. 20% KOH.

in the organisms was maintained for at least 48 hr. after cessation of growth. Organisms collected from cultures with limiting concentrations of nicotinic acid contained about 30–40 $\mu\text{g.}$ thiamine/g. dry wt. organisms at the end of the linear growth phase, and during 12 hr. after the cessation of growth the content fell to 10–12 $\mu\text{g.}$ thiamine/g. dry wt. organisms. When cultures were grown with limiting nicotinic acid concentrations but with 0.2 $\mu\text{g.}$ thiamine/ml. present initially, the thiamine content of the organisms remained high during and after the linear phase of growth. In a culture of this kind, the concentration of thiamine in the organisms after 48 hr. was 60 $\mu\text{g./g.}$ dry wt. organisms. Thiamine estimations on organisms and supernatant fluids from the culture showed that the total organism + supernatant fluid thiamine amounted to only c. 35% of that initially added. The thiamine concentration in the supernatant fluid of the centrifuged culture at the end of the experiment was 0.035 $\mu\text{g./ml.}$

Thiamine synthesis during lag phase

Organisms which contained initially 15 $\mu\text{g.}$ thiamine/g. dry wt. were suspended in fresh medium, with an excess of nicotinic acid, to a concentration equivalent to 0.26 mg. dry wt. organism/ml. Pyruvate accumulation and opacity changes were followed during the lag phase, and a sample of organisms for thiamine estimation was collected when the pyruvate concentration was falling and growth was just beginning. During the lag phase the thiamine content of the organisms rose from the initial 15 $\mu\text{g.}/\text{g.}$ dry wt. organism to 40 $\mu\text{g.}/\text{g.}$ dry wt.

Thiamine uptake by cells

Organisms were collected from cultures which had been grown for 48 hr. with a limiting concentration of nicotinic acid (0.02 $\mu\text{g.}/\text{ml.}$) and with an excess of nicotinic acid. They were re-suspended in 10 ml. vol. of 0.2M-phosphate buffer (pH 7.3) to a concentration equivalent to 1.8 mg. dry wt. organisms/ml. and 2 ml. of a thiamine solution containing 1 $\mu\text{g.}$ thiamine/ml. was added to each. The suspensions were shaken at 37° for 10 min. and were then centrifuged. The organisms were washed three times with glass-distilled water and their thiamine content determined. Simultaneous thiamine estimations were made on samples from the same cultures which were not exposed to thiamine after collection. The thiamine content of the organisms grown with an excess of nicotinic acid was 50 $\mu\text{g.}/\text{g.}$ dry wt. organism and this was not increased by exposure to thiamine in the concentration used. The thiamine content of the organisms grown with limiting nicotinic acid concentration was 12 $\mu\text{g.}$ thiamine/g. dry wt. organism and after exposure to thiamine this rose to 55 $\mu\text{g.}/\text{g.}$ dry wt. organism.

Effects of incubation with lactate on thiamine content of organisms

Three 30 ml. samples (A, B, C) of a culture which had grown for 24 hr. with excess of nicotinic acid were collected and centrifuged. The thiamine content of sample A was estimated at this stage. The sample B was resuspended in 40 ml. 0.05M-phosphate buffer (pH 7.4)+10 ml. water, and was incubated overnight on the shaker. Sample C was resuspended in 40 ml. 0.05M-phosphate buffer (pH 7.4)+10 ml. 0.5M-lactate and incubated overnight on the shaker. The organisms from suspensions B and C were collected and their thiamine content determined for comparison with A. The thiamine concentrations found were ($\mu\text{g.}$ thiamine/g. dry wt. organism), respectively: A, 33; B, 30; C, 19. Pyruvate had accumulated in C.

Crystallization of pyruvic 2:4-dinitrophenylhydrazone from cultures

Pyruvic 2:4-dinitrophenylhydrazone could easily be crystallized from culture medium which had accumulated 3 mg. pyruvate/ml. To 400 ml. culture fluid after centrifugation was added about 90 % of the calculated required amount (on the basis of pyruvate content of the medium) of concentrated 2:4-dinitrophenylhydrazone in 2N-HCl; the pyruvic 2:4-dinitrophenylhydrazone was precipitated. The precipitate was collected on a sintered

glass filter and was washed twice with 2N-HCl. The crude pyruvic 2:4-dinitrophenylhydrazone was dissolved in a minimal amount of ethyl acetate and transferred to a separating funnel. The ethyl acetate solution was extracted with a minimal amount of 10% (w/v) sodium carbonate solution, and the sodium carbonate layer collected. The pyruvic 2:4-dinitrophenylhydrazone was precipitated from this by slow addition of 4N-HCl, and the precipitate was collected by filtration using a sintered glass filter. The precipitate was washed 5 times with N-HCl, redissolved in a minimal quantity of warm ethyl acetate, heated cautiously over a water-bath, and when it had dissolved, the solution was filtered through a Whatman no. 54 filter paper. Crystallization occurred from the filtrate. The solid material was recrystallized several times from ethyl acetate and was dried *in vacuo* over H_2SO_4 . The melting-point was found to be 217° ; a mixed melting-point with a known pure sample was the same. The nitrogen content corresponded to the expected value of 20.9%.

Results with other strains of Proteus vulgaris

Five other nicotinic acid-requiring strains of *Proteus vulgaris* were investigated; in all of them similar phenomena occurred during the lag phase and linear and terminal growth phases.

DISCUSSION

The accumulation of pyruvate during the phase of linear growth in cultures of *Proteus vulgaris* with growth-limiting nicotinic acid concentrations is quickly reversed by the addition of more nicotinic acid or coenzyme 1 (diphosphopyridine nucleotide). The organisms at this time are not strikingly deficient in thiamine, although their content is *c.* 10–15 $\mu\text{g./g.}$ dry wt. organism below that found in cultures grown with excess nicotinic acid. The presence of 10^{-5}M -thiamine in the medium initially does not prevent pyruvate accumulation during the linear phase, and under these conditions the thiamine content of the organisms exceeds 50 $\mu\text{g./g.}$ dry wt. organism. Throughout the linear growth phase the concentration of nicotinic acid in the developing mass of organisms must be diminishing, since all the available nicotinic acid is in the organisms and at the same time growth is continuing (Jackson & Copping, 1952). The thiamine concentration in the organisms remains in excess of 30 $\mu\text{g./g.}$ dry wt. organism even in the absence of added thiamine.

In the stationary phase, the organisms which are too deficient in nicotinic acid for growth to occur are in contact with a medium in which the concentration of pyruvate is steadily increasing. Under these conditions, the organisms become thiamine-deficient and the addition of nicotinic acid alone to a pyruvate-containing suspension of washed organisms which are deficient in both nicotinic acid and thiamine is followed by a marked increase in rate of oxygen uptake. However, when thiamine or cocarboxylase is added with nicotinic acid the rate of oxygen uptake is increased still further and may be double that observed with nicotinic acid alone. The addition of thiamine or cocarboxylase alone causes only a slight increase in rate of oxygen uptake.

These effects of added thiamine or cocarboxylase are not seen with organisms collected at the end of the linear phase of growth or early in the stationary phase, when the cell concentration of thiamine in the organisms is about 30 $\mu\text{g./g.}$ dry wt. organism. The effects of thiamine or cocarboxylase on the lag phase of organisms which have been washed and transferred to fresh medium parallel the effects on oxygen uptake. The lag phase experiments emphasize the fact that, in these cultures, the onset of growth is related to the increased ability of the organisms to utilize pyruvate, which is dependent on adequate concentrations of thiamine and nicotinic acid in the organisms.

The development of thiamine deficiency during the stationary phase within a similar period does not occur when lactate, and not nicotinic acid concentration, is limiting growth. Under these conditions the lactate is completely utilized, and the organisms do not become deficient in nicotinic acid. Accumulation of pyruvate does not occur since there is not an excess of lactate during the stationary phase, and the organisms do not remain in contact during this phase with a pyruvate solution.

During the lag phase of thiamine-deficient organisms in the absence of added thiamine but with added nicotinic acid, thiamine is synthesized by the organisms and growth does not occur until concentrations of thiamine of *c.* 40 $\mu\text{g./g.}$ dry wt. organisms have been reached. As thiamine is synthesized, the ability to metabolize pyruvate is increased, and the pyruvate which has accumulated disappears, growth beginning when the pyruvate concentration in the medium has fallen back to about 10 $\mu\text{g./ml.}$

When the thiamine-deficient organisms are resuspended in fresh medium with nicotinic acid, they must synthesize thiamine before growth can occur. When thiamine is added, it can quickly be taken up by the thiamine-deficient organisms, and concentrations of thiamine in the organisms similar to those found in cultures growing with excess nicotinic acid are reached within a short time, perhaps seconds or at most a few minutes, even on exposure to low external concentrations of thiamine. At least some of this added thiamine is quickly converted to a functional form, as shown by the rapid decrease in pyruvate concentration in the medium. It seems that the thiamine-deficient organisms when supplied with sufficient nicotinic acid can promptly phosphorylate added thiamine.

The pyruvic oxidase of *Proteus vulgaris* described by Stumpf (1945) was apparently a complex consisting of a specific protein, diphosphothiamine (cocarboxylase) and a bivalent metal probably magnesium. The intact organisms contained a phosphatase which split diphosphothiamine to thiamine. Moyed & O'Kane (1956) showed that in *P. vulgaris* pyruvate was oxidized by a soluble cocarboxylase-dependent dehydrogenase which was coupled with an auto-oxidizable cytochrome system; coenzyme A, orthophosphate, lipoic acid and flavine-adenine-dinucleotide were not involved. Neither Stumpf nor Moyed & O'Kane found that coenzyme I was necessary for pyruvate oxidation. The strains used in the present investigation had the same cytochrome components as those described by Moyed & O'Kane. The thiamine concentrations in the organisms which were necessary for optimal pyruvate utilization

have been found, but the cocarboxylase concentrations in the organisms were not measured. Even with added thiamine or cocarboxylase, washed suspensions of nicotinic acid-deficient *P. vulgaris* have a low pyruvic oxidase activity. Perhaps in the whole organisms the cocarboxylase-dependent system can link with a cozymase-dependent system. It is possible, too, that in the intact organisms there is a continuous breakdown of cocarboxylase to thiamine by the phosphatase present, and that in the absence of sufficient nicotinic acid the resynthesis of cocarboxylase is impaired. Added cocarboxylase does not, however, prevent pyruvate accumulation during the linear growth phase, and itself produces only a slight increase in rate of pyruvate oxidation by older organisms.

Thanks are due to Miss L. Hopkinson and Mr R. W. Warner for technical assistance in this work. Dr D. Herbert kindly supplied a pure sample of pyruvic 2:4-dinitro-phenylhydrazone.

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A Study of Antigenic Variation in *Vibrio cholerae*

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SUMMARY: The variations in *Vibrio cholerae* studied included changes from Ogawa to Inaba type, smooth→rough, and motile→non-motile. With a sloppy-agar technique it was possible to estimate the rate of formation of these variants (i.e. probable mutation rate 1 in 10^5 and 1 in 10^4 , respectively). It was possible to show that the action of antiserum in promoting the change from Ogawa to Inaba was selective rather than mutagenic. With the other two variations studied no selective process had to be considered owing to their high spontaneous rate of appearance. It may be said that all three variations studied were due to spontaneous mutants in the parent cultures giving rise to these forms.

The studies of Gardner & Venkatraman (1935) showed that vibrio species could be separated on the basis of their somatic 'O' antigens, while the flagellar 'H' antigens were widely shared. Since then the differentiation of *Vibrio cholerae*, the pathogen of cholera, from similar cholera-like vibrios, rests on its agglutinability with specific 'O' antisera. *V. cholerae* constituted O group I, in this scheme of classification, along with certain haemolytic 'El Tor' vibrios. The definition of *V. cholerae* as a non-haemolytic vibrio belonging to O group I (Gardner & Venkatraman) is now generally accepted, and O antisera prepared with heat-killed suspensions are almost universally used for the identification of *V. cholerae*, in the absence of any reliable biochemical criteria. Gardner & Venkatraman (1935) also confirmed the observations of early Japanese workers (Kabeshima, 1918; Nobechei, 1923) when they defined subtypes Inaba, Ogawa and Hikojima in *V. cholerae* which, while sharing group-specific O antigens, differed in minor type-specific O antigens. Types Inaba and Ogawa were the 'end' types, usually designated by the antigenic symbols AC and AB respectively, while Hikojima formed the intermediate type ABC. In view of the poor content of antigen C in Hikojima strains, as well as its presence in Ogawa type cultures when grown at 20°, Kauffmann (1950) suggested that Ogawa-Hikojima types constituted one form of *V. cholerae*, as distinct from the Inaba form.

Cholera outbreaks in India are usually confined to a single type of *Vibrio cholerae* in localized areas, though the type may vary from place to place and epidemic to epidemic. Hikojima-type strains seem to be of rare occurrence while Inaba and Ogawa types have contributed to most epidemic outbreaks. The shift between these two types, over several years, in the cholera endemic

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areas of Madras State was summarized by Venkatraman (1947), who observed that a change in antigenic type followed years of comparatively low incidence of cholera.

Changes in antigenic type of *Vibrio cholerae* under laboratory conditions were observed by Kabeshima (1918) and a detailed study of this phenomenon was made by Shrivastava & White (1947). They observed that Inaba-type organisms could be consistently isolated from Ogawa-type cultures when the latter were grown in broth containing type-specific Ogawa O antiserum. A similar expedient of growing Inaba-type cultures in broth containing Inaba O antiserum yielded only rough forms and not the Ogawa type, except in the case of certain imperfect Inaba strains which appeared to belong in reality to the Hikojima category. This evidence of a one-way antigenic change in *V. cholerae* probably led Shrivastava & White (1947) to believe that they resulted from loss variations, presumably through some fault in germinal distribution on cellular division. The present investigation was designed to study the change of Ogawa to Inaba type and certain other antigenic changes, with the aid of a rapid technique which permitted the typing of a large number of colonies at a time without resorting to laborious agglutination tests.

METHODS

Media. For the cultivation of *Vibrio cholerae*, the PLYP medium described by Gorrill & Gray (1956) was used. This contained peptone (Evans), 10 g.; Yeastrel (Brewers Food Supply Co. Ltd., Edinburgh, 3), 3 g.; Lab-Lemco, 5 g.; Pronutrin (Herts Pharmaceuticals Ltd., Welwyn Garden City), 10 g.; and sodium chloride (AR), 5 g.; in 1 l. distilled water. The mixture was adjusted to pH 8.0 and autoclaved at 10 lb./sq.in. for 10 min. Difco agar to 2% (w/v) was added to make solid medium when required.

Strains of Vibrio cholerae used. Inaba-type cultures: 243, King Institute of Preventive Medicine, Madras; 8024, National Collection of Type Cultures, Colindale Avenue, London, N.W. 9; 53/54 and 55/54, All India Institute of Hygiene and Public Health, Calcutta.

Ogawa-type cultures: 502, Central Drug Research Institute, Lucknow; M. 64, M. 70, M. 76, All India Institute of Hygiene and Public Health, Calcutta; 8022, National Collection of Type Cultures, Colindale Avenue, London, N.W. 9.

Antisera. High-titre agglutinating O antisera were prepared by immunizing adult male rabbits with heat-killed cultures of *Vibrio cholerae*, as carried out by Gardner & Venkatraman (1935). Monospecific Inaba and Ogawa O antisera were obtained by absorbing the corresponding sera with heat-killed cultures of the heterologous type. The agglutinin titres of the antisera varied from 1/2500 to 1/5000. The rabbits were re-immunized on five or six successive occasions at 2- to 3-monthly intervals with the respective antigens. The O agglutinin titre was maintained by this means and in a few animals the titre went up to 1/10,000 after repeated immunization.

Procedure for typing colonies of Vibrio cholerae. The antiserum in sloppy-agar technique used by Stocker (1949) to differentiate antigenic phases in *Salmo-*

nella typhimurium proved very satisfactory for the differentiation of antigenic types in *V. cholerae* and will be referred to as 'Stocker's sloppy-agar technique'. The test, performed as below, gave consistent results:

(i) PLYP medium containing 2% Difco agar, and sloppy PLYP medium containing 0.5% Difco agar were prepared. The sloppy medium was dispensed in 10 ml. amounts in 1 oz. screw-capped bottles, while the solid medium was poured into 9 cm. diam. Petri dishes, using about 15 ml. medium/dish.

(ii) The culture to be tested was seeded on the agar plate at such a dilution as to give about 200 to 400 colonies/dish.

(iii) When fully developed colonies appeared on the medium after 18–24 hr. of incubation at 37° the test was performed. To 10 ml. of sloppy agar, melted in the steamer and cooled to 50°, was added 0.1 ml. of type-specific antiserum (either Inaba or Ogawa) and after mixing well, gently layered on the surface of the culture in the dish. After allowing 10 min. for the agar to set, the plates were transferred to an incubator (37°) and results read after 15 min.

Three different colony appearances were encountered:

(a) 'haloed' colonies: these colonies appeared dense and opaque with a well-pronounced halo round each colony as the organisms diffused out. This appearance was associated with types not agglutinated by the serum used.

(b) 'non-haloed' colonies: these colonies appeared dense and opaque but without a halo. The edge of the colony was clear-cut. This appearance was found with types agglutinated by the serum used.

(c) 'compact' colonies: these colonies differed from the non-haloed type in being thin and translucent. The dense opalescence did not develop on the addition of sloppy agar, nor was there a halo around the colony. This appearance was found in non-motile mutants and in salt-unstable rough mutants. Colonial differentiation between these two was not possible by this method.

The reliability of the above appearances and their correlation with the associated characters was checked by model experiments with type mixtures. The appearance of rough and non-motile mutants in this test was not influenced by the antiserum in the sloppy layer. They showed the same characteristics in antiserum-free sloppy agar which served to differentiate them from smooth motile organisms, and was in fact used to isolate such mutants. When using antiserum in the sloppy layer to differentiate between the types, it was essential to have an adequate concentration of the antiserum. With titres of 1/2500 or more, 0.1 ml. of undiluted antiserum for 10 ml. of sloppy agar was sufficient. Antisera with lower titres were not used in this test.

RESULTS

Change from Ogawa to Inaba type

If change from Ogawa to Inaba type were to occur by mutation, as suggested by Shrivastava & White (1947), it was essential to exclude any possibility that the antiserum used had any role other than the mechanical separation of the parent organisms from the mutants, by clumping the parents and allowing the mutants to multiply in the supernatant fluid. Before proceeding to test

this, preliminary experiments were carried out on the same pattern as those of Shrivastava & White (1947) to confirm this change with strains Ogawa 502 and Ogawa M 64. The results of these experiments are recorded in Tables 1 and 2.

Table 1. *Ogawa*→*Inaba* transition in *Vibrio cholerae* strain Ogawa 502

A culture of *V. cholerae*, strain Ogawa 502, was made in 5 ml. broth containing 1/50 Ogawa O antiserum (titre 1/2500). The supernatant fluid was plated out at intervals during incubation at 37° to give discrete colonies.

Inoculum size (no. of organisms)	Time at which sample was plated											
	2 hr.			4 hr.			7 hr.			24 hr.		
	No. of colonies	Type of colony*			No. of colonies	Type of colony			No. of colonies	Type of colony		
		In	Og	r		In	Og	r		In	Og	r
8×10^9	80	—	80	—	80	1	79	—	80	—	79	1
8×10^7	80	—	80	—	80	—	80	—	80	—	80	—
8×10^5	80	—	80	—	80	—	80	—	80	—	80	—
8×10^3	80	—	80	—	80	—	80	—	80	—	79†	—

* Colony type: In = *Inaba*; Og = *Ogawa*; r = rough.

† 1 non-motile.

Table 2. *Ogawa*→*Inaba* transition in *Vibrio cholerae* strain Ogawa M 64

A culture of *V. cholerae*, strain M64 in 5 ml. broth containing 1/50 Ogawa O serum (titre 1/2500). Supernatant fluid plated out at intervals for discrete colonies (see Table 1).

Inoculum size (no. of organisms)	Time of plating during incubation at 37°											
	2 hr.			4 hr.			7 hr.			24 hr.		
	No. of colonies	Type of colony			No. of colonies	Type of colony			No. of colonies	Type of colony		
		In	Og	r		In	Og	r		In	Og	r
3.5×10^9	40	—	40	—	40	—	40	—	40	40	—	—
3.5×10^7	40	—	40	—	40	—	40	—	40	39	1	—
3.5×10^5	40	—	40	—	40	—	40	—	41	1	8	32
3.5×10^3	40	—	40	—	40	—	40	—	40	—	9	31

These experiments studied the effect of decreasing size of inoculum and the period of incubation in Ogawa-antiserum broth required for the isolation of *Inaba* mutants. Twenty-four hr. cultures of the strains were suspended in saline, and 100-fold dilutions made in broth. From the undiluted suspension and from each dilution 1 ml. samples were transferred to 5 ml. broth containing 1/50 Ogawa O antiserum (titre 2500) and the supernatant fluid plated out to give discrete colonies after different periods of incubation. The types of colonies thus obtained were tested by Stocker's sloppy-agar technique, with Ogawa O antiserum in the sloppy layer. Mutants which were isolated by this method were checked later by slide-agglutination tests. 'Compact' colonies were re-examined to ascertain whether they were rough or non-motile mutants.

The supernatant cultures of both strains after incubation for 24 hr. were predominantly of the Inaba type when the inoculum was greater than 10^7 organisms. When the inoculum was 10^5 organisms, Inaba mutants did not appear with Ogawa 502 (Table 1) while only one of 41 colonies was of the Inaba type in Ogawa M 64 (Table 2). With an inoculum of 10^3 organisms, Inaba mutants did not appear with either strain. The frequency of appearance of rough mutants was in the reverse order, being most frequent with the smaller inocula. Further, the period of incubation was important as only a few mutants could be isolated from earlier samples, but they predominated after 24 hr.

These findings, though not conclusive, are consistent with the mutation hypothesis. With larger inocula mutants were probably introduced into the culture at the start and the time-lag indicated the period required for mutants to become predominant in the supernatant culture. It was also possible that smaller inocula, which were presumably devoid of mutants at the time of implant, grew in clumps in the depths of the medium under conditions unfavourable for active multiplication and for the expression of mutants. The predominance of rough mutants with the smaller inocula appeared to indicate that this mutation was more frequent than the change Ogawa→Inaba. The apparent absence of rough forms in the presence of Inaba mutants is difficult to explain and was probably due to smooth and rough strains of *Vibrio cholerae* having different growth rates as observed by Bhaskaran (1953).

Proof of the presence of Inaba mutants in Ogawa cultures before serum treatment would require their detection in the parent culture. Judging from these experiments this might be possible if at least 10^7 colonies of the parent culture were examined. As this was not practicable it was necessary to resort to procedures which might be expected to simplify the study.

Treatment with ultraviolet irradiation. As ultraviolet irradiation is known to increase mutation rates in bacteria, this treatment was used to see whether Inaba mutants could then be isolated from Ogawa cultures without the aid of antiserum. A volume (10 ml.) of a saline suspension of strain Ogawa 502 was exposed to u.v. radiation (10 ergs./mm.²/sec.) for 3 min., after which 0.3 ml. of the irradiated culture was grown in broth for 4 hr. to allow the phenotypic expression of the mutants. The culture was then plated out on several agar plates to give discrete colonies and each colony thus obtained was typed by Stocker's sloppy-agar method. It was possible in this way to examine a total of 10,812 discrete colonies; of these 4 were rough, the rest being of the parent Ogawa type.

Treatment with antiserum in the cold. As the above technique failed to show Inaba mutants in the Ogawa cultures, it was necessary to revert to antiserum as a selective agent, but the experiments were now carried out in the cold to prevent the growth of the organisms, while a partial separation of the parent from mutant organisms was produced by agglutinins present in the antiserum. Pre-existing mutants would escape agglutination in the culture examined, and their numbers in relation to the colony count would indicate the approximate mutation frequencies.

Table 3 records the results of experiments carried out in this way with three Ogawa strains of *Vibrio cholerae*. The test was carried out by adding 0.1 ml. Ogawa O antiserum (titre 1/2500) to 5 ml. cold saline suspensions of each culture and keeping these in the refrigerator for 2 hr. The clumps were separated by spinning at 2500 rev./min. for 30 min. in a refrigerated centrifuge. The supernatant fluid was then plated out to give discrete colonies. From the colony counts of the original culture and the supernatant fluid, and from the proportion of Inaba organisms detected in the latter, it was possible to estimate the approximate number of Inaba mutants in the culture. Table 3 shows that the population was decreased from 10^9 organisms/ml. to 10^5 or 10^6 organisms/ml. as a result of agglutination with antiserum and subsequent centrifugation. The number of Inaba mutants isolated in relation to the number of colonies examined from the supernatant fluid indicated that in the original culture Inaba mutants could not be present at less than 1 in 4.8×10^6 , 1 in 1.3×10^7 , and 1 in 10^7 organisms in the three strains examined.

Table 3. *Detection of Inaba mutants in Ogawa type cultures of Vibrio cholerae*

Strains	Colony count		No. of colonies examined	No. of mutants		Frequency of mutant in parent culture
	Original culture	Supernatant fluid				
Ogawa M64	1.6×10^9 /ml.	1.3×10^6 /ml.	7800	2 Inaba	1 Ogawa (non-motile)	$1.4.8 \times 10^6$
Ogawa M70	1.9×10^9 /ml.	2.4×10^5 /ml.	5000	4 Inaba	1 rough	1.1×10^7
Ogawa M76	2.6×10^9 /ml.	6.5×10^5 /ml.	3250	1 Inaba	6 rough	$1.1.3 \times 10^7$

There was scope for error in these estimates. It was inevitable that during centrifugation some of the mutant organisms might be deposited along with the agglutinated clumps of the parent culture. The extent to which this occurred was revealed by model experiments, carried out under identical conditions, and using a mixture of known viable populations of the two types. This showed that only 2% of the Inaba organisms in the mixture was accounted for. A source of error even in these model experiments was the almost certain presence of Inaba mutants introduced with the Ogawa culture, which could not be taken into account. These facts emphasized that estimates of the ratio mutant:parent, as recorded in Table 3, could only be approximate even when a correction was applied on the basis of the model experiments. With the correction ($50 \times$) the approximate number of Inaba mutants in Ogawa cultures would be 1 in 10^5 in strain Ogawa M 64, 1 in 2×10^5 in strain Ogawa M 70, and 1 in 2×10^5 in Ogawa M 76.

Possibility of Inaba to Ogawa mutation

Shrivastava & White (1947) observed that a change from Inaba to Ogawa could not be demonstrated in Inaba strains under conditions similar to those which facilitated the isolation of Inaba mutants from Ogawa cultures, that is,

by growing them in broth containing type-specific antiserum. This question was re-examined, as it was possible that with Stocker's sloppy-agar technique the change might be demonstrable. Experiments were carried out with four Inaba strains: Inaba 8024, Inaba 53/54 and Inaba 55/54 (wild strains) and strain B 40 an Inaba mutant of Ogawa M 70 isolated in a previous experiment. When these strains were grown in broth containing Inaba O antiserum for 24 hr., and the supernatant fluid plated out to give discrete colonies, numerous rough colonies were seen but none was of the Ogawa type. With the intention to decrease the predominance of rough variants, in one experiment 'rough' antiserum was also added to the broth. This decreased the number of rough colonies obtained from the supernatant fluid but did not reveal Ogawa-type colonies. Serial cultivations in these antiserum media did not result in Ogawa mutants either.

Rough and non-motile mutants of Vibrio cholerae

The Stocker sloppy-agar technique, while applicable to type determination of *Vibrio cholerae*, could also be used for the identification of rough and non-motile mutants. With antiserum-free sloppy agar 4 smooth motile strains were examined for the frequency of appearance of rough or non-motile colonies. Table 4 shows that rough and non-motile mutants appeared with a greater frequency than the antigenic-type mutations studied earlier, particularly in strain Inaba 243 (1 rough mutant in every 1300 colonies and 1 non-motile mutant in every 2000 colonies). The frequency of these mutations in the other strains varied from 1 in 1.4×10^4 to 1 in 1.2×10^4 for non-motile mutants, and from 1 in 9×10^3 to 1 in 4×10^3 for rough mutants. From these findings it seems safe to conclude that these mutations were of the order of 1 in 10^4 divisions or less.

Table 4. *Rough and non-motile mutants of Vibrio cholerae*

Strain	No. of colonies examined	No. of rough mutants	No. of non-motile mutants
Inaba 8024	9,100	1	0
Inaba 243	12,000	9	6
Ogawa 502	14,200	2	1
Ogawa 8022	12,000	3	1

DISCUSSION

Antigenic variants of *Vibrio cholerae* have been demonstrated in the presence of immune serum or phage (Yang & White, 1934; Shrivastava & White, 1947). It is often inferred that these agents induced the observed changes in the organism. References in the literature to 'converted' Inaba strains (meaning Inaba strains isolated from Ogawa cultures) are instances of this belief. Shrivastava & White (1947) were, however, careful to point out that any attack on the surface of a cell could not be expected to induce transmissible modifications. They also indicated that these changes might be due to loss variations during growth. The basic difficulty in determining whether a change

is due to mutation or not, is the difficulty of examining a large number of bacteria for the existence of organisms with the variant character without using selective methods which might act directly on the organisms. If this difficulty could be overcome, their mutational origin could be established. It was with this in view that the sloppy-agar technique described by Stocker (1949) was adapted for the present study. Type differentiation of a large number of colonies was possible by this means, and in all three cases the time of exposure to the antiserum in the sloppy agar was too short to induce variants. These studies established that the rough and non-motile variants were always present in the parent culture and hence were due to mutations, which appeared more or less with the same frequency in each case (about 1 in 10,000 colonies examined). It may be mentioned that Yang & White (1934) were successful only once in demonstrating the existence of rough mutants in smooth cultures by the ingenious procedure of washing plated cultures of smooth strains with 2% NaCl solution and examining the adherent colonies which survived the treatment. As regards non-motile mutants, their direct demonstration in populations of motile strains of *Vibrio cholerae* has not hitherto been reported. However, the existence of such strains was known; Balteanu (1926) found that they were associated with opaque colony character.

Unequivocal evidence for antigenic type variation in *Vibrio cholerae* as a result of mutation could not be obtained. However, experiments with antisera carried out in the cold confirmed that growth of the culture subsequent to the addition of specific antiserum was not necessary for the isolation of Inaba mutants from Ogawa cultures. This makes it very probable that these mutants were already present in the parent culture, and agglutination by antiserum only facilitated isolation of the mutant in the supernatant fluid. The rate of appearance of these mutants was of the order of 1 in 10^5 cell-divisions. The inability to demonstrate directly the presence of these mutants in Ogawa cultures was probably due to this low mutation rate, which would require the examination of a similarly large number of colonies for a chance isolation of the mutant. This was obviously not possible even with the sloppy-agar technique.

The failure to isolate Ogawa mutants from Inaba cultures with the aid of selective antiserum, may have been the result of still lower reverse mutation rates. Further, only single-step mutations can be revealed by the tests used and perhaps the change Inaba→Ogawa type involves multi-step mutation. The occurrence of Hikojima type, intermediate between Inaba and Ogawa, as well as the identification of imperfect Inaba strains (containing demonstrable Ogawa antigenic factor) by Shrivastava & White (1947), may be of significance in this connexion. It would be interesting to inquire into the origin of these Hikojima strains. Either they occurred as a result of a partial carry-over of Ogawa antigen in Inaba mutants, or they were due to a reverse change from Inaba to Ogawa type by successive mutations, accompanied by a progressive replacement of the C antigen of Inaba by the B antigen of Ogawa. Proof of this might be possible if such intermediate types could be isolated from pure Inaba or Ogawa strains; in the absence of any selective method this may be

difficult. This multi-step mutational hypothesis may also explain the occurrence of strains of trivalent agglutinability with Inaba, Ogawa and 'rough' antisera (Shrivastava & White, 1947; Bhaskaran, 1953).

The loss-variation hypothesis of Shrivastava & White (1947) is an alternative explanation for the non-reversion of Ogawa to Inaba mutation. They suggested that the transition from Ogawa to Inaba type may be due to failure of the organism to synthesize certain chemical groupings, so that the reverse change would be one of synthetic revival which might be difficult to achieve. No differences were, however, seen in the nutritional requirements of Ogawa and Inaba type strains of *Vibrio cholerae* (Bhaskaran & Rowley, 1956), though the differences may not be so gross as to be revealed by the auxanographic methods used in that study, which showed a general requirement for purines. More detailed knowledge of the synthesis of purines by *V. cholerae* and the role of certain amino acids which are essential nutrients for some Inaba strains, may show whether there is any difference in this aspect of the synthetic processes in the two types.

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Multiplication of the Virus of Foot-and-Mouth Disease in Culture

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SUMMARY: The infectivity curve of the virus of foot-and-mouth disease was studied in cultures of suspended trypsinized cattle-tongue epithelial cells. By this technique a known number of cells could be exposed to a known amount of virus. The pattern of multiplication was that adsorption of the virus by the cells occurred rapidly and, except with low concentrations of virus, was complete in 15-30 min. A latent period of about 2.5 hr. followed during which the virus was closely associated with the cells, was protected from the neutralizing action of antiserum, was not readily extractable but retained its infectivity. At the end of the latent period the infected cells became producers of virus at a rate estimated to be between 10^2 and 10^3 ID₅₀/15 min. At about 12 hr. the infectivity of the culture began to decline because of death of virus-producing cells and thermal inactivation of the virus.

The *in vitro* cultivation of a virus in isolated tissue offers many advantages for studying its multiplication compared with attempts to do so in an infected host. Maitland & Maitland (1931) and Hecke (1930, 1931) reported the successful *in vitro* cultivation of the virus of foot-and-mouth disease in guinea-pig embryo tissue. Frenkel (1947, 1953) developed this culture method for routine vaccine production with epithelial tissue of cattle tongue. A variety of methods of cultivation have been studied at this Institute for virus production, for studying virus multiplication and for the development of methods of virus titration. The present paper gives the results of virus multiplication studies with culture of suspended trypsinized cattle-tongue epithelial cells. This was a development of preliminary studies of the multiplication of this virus in suspended fragments of tongue epithelium. The curves obtained as a result of infectivity titrations of tissue fragments and of the culture liquid were described by a number of workers (Henderson, 1953*a, b*; Brooksby & Wardle, 1954; Mackowiak, Girard, Camand & Hirtz, 1955; Ubertini, Nardelli, Barei & Santero, 1956). There is agreement about their general form but modifications in method were obviously necessary before a more detailed examination could be made. The techniques described in this paper, by means of which a known number of epithelial cells were exposed to a known amount of virus, were evolved for this purpose. For ease of description, it is convenient to identify the following portions of the infectivity curve; namely, the adsorption period, the latent period, the period of increasing infectivity, the period of decreasing infectivity.

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METHODS

Foot-and-mouth disease virus. Strain Ven 1, Vallée O type. This strain was recovered from material received from an outbreak of the disease at Villa de Cura, Venezuela, in June 1950. Cultivation was begun with virus of the 2nd cattle passage in 1950; this line has since been maintained solely in culture.

Foot-and-mouth disease antiserum. Antiserum was collected from cattle or from guinea-pigs hyperimmunized with strain Ven 1 virus. The potency of the serum was determined by neutralization tests in unweaned mice (Skinner, 1953).

Preparation of tissue. Epithelial tissue was collected in strips from the tongues of recently slaughtered cattle by means of a meat-slicing machine. For routine passaging and preparation of seed virus for experiment, these strips of tissue were clipped with scissors into fragments about 5 mm. square. The tissue was used within 3 days of collection, being stored in the interim at 4° in Tyrode solution.

Trypsinized cell suspensions were prepared from strips of tongue epithelial tissue by digestion in a 0·5% (w/v) solution of trypsin (1/250; Difco Laboratories, Detroit, Michigan) in a modified Tyrode solution free from Ca and Mg (Rinaldini, 1954; pH 7·6) for up to 2 hr. at 37°. The tissue was stirred throughout this incubation. The undigested tissue and large clumps of cells were allowed to sediment and the supernatant cell suspension pipetted off into weighed bottles, centrifuged and the supernatant discarded. The deposit was resuspended in normal bovine serum and held for 10–15 min. at 37° to allow the antitryptic action of the serum to operate. This suspension was then centrifuged, the supernatant discarded, the bottle weighed and the deposit resuspended with culture medium to a concentration of 10% (w/v). Cell counts were made of this final suspension by mixing with an equal volume of 2% eosin in Tyrode solution and using a standard haemocytometer chamber. Ability to stain with eosin was taken as an indication that an epithelial cell was non-viable (Schrek, 1936).

Culture media. For routine passaging the medium used was Tyrode solution + 10% (v/v) bovine serum or + the amino acids suggested by Frenkel (see Girard & Mackowiak, 1953). Bovine amniotic fluid or Tyrode solution + 0·5% (w/v) lactalbumin hydrolysate (Nutritional Biochemicals Corporation, Cleveland, Ohio) was used for cell suspension cultures, this latter medium being employed for all cultures demanding only survival of suspended cells. All media had added to them 200 µg. dihydrostreptomycin sulphate/ml. 200 units benzylpenicillin (sodium salt)/ml. and 0·001% (w/v) phenol red. Since the year 1955, 20 units 'Mycostatin' (E. R. Squibb and Sons, London and New York)/ml. have been added to control the growth of yeast contaminants when the culture was to be incubated for longer than about 12 hr. (Wigmore & Henderson, 1955). The above media were found to be more satisfactory for survival of cells and multiplications of virus than Tyrode solution; Tyrode solution + 1% (v/v) solution of protein hydrolysate (veterinary; Wellcome Research Laboratories, Beckenham, Kent), glucosol phosphate solution or Simm's Z16 solution (Parshley & Simms, 1950).

Glassware. With epithelial cell suspension cultures difficulty was experienced initially because the cells stick to the glass of culture bottles. This was intensified by the use of silicones but was effectively minimized by coating the glass with paraffin wax (m.p. 56°). The wax was removed after the bottles had been used by rinsing with hot water. Glassware used in preparing cell suspensions goes through a special procedure used at this Institute for tissue culture glassware; namely, soaking in 10% (v/v) hydrochloric acid if tissue debris cannot be removed by brushing, rinsing in tap water, autoclaving in tap water + 'Calgon' water softener (Albright and Wilson, Ltd., London) + 0.1% (w/v) sodium lauryl sulphate ('Empicol LZ' powder, Marchon Products Ltd., Whitehaven, Cumberland), rinsing in tap water, rinsing in distilled water, autoclaving in distilled water, rinsing in double-distilled or de-ionized distilled water and sterilizing in the autoclave or hot air oven (Sellers, unpublished work). Glassware used in routine passaging of the virus, preparation of dilutions, etc., is dealt with by standard laboratory methods.

Culture methods. Seed virus filtrates are prepared from suspensions of ground tissue in equal parts M/25 phosphate buffer solution and Hartley's digest broth (pH 7.6) using filter pads of Seitz EK porosity.

For routine passaging the method used was a modification of that described by Frenkel (1947). Clipped tongue epithelium was added to the seed virus filtrate and stored at 4° for not less than 1 hr. The tissue was then added to 50 ml. volumes of medium in 725 ml. flasks. The air in the flask was replaced with a mixture of 95% (v/v) oxygen + 5% (v/v) carbon dioxide and the flask sealed. These flasks were incubated at 37° for 18–24 hr., being gently rocked during this time. This was the method used in the preliminary studies of the multiplication of the virus of foot-and-mouth disease in culture except that the tissue was washed before it was added to the culture medium by repeated centrifugation in Tyrode solution (Henderson, 1953*a, b*).

The technique used for cell suspension cultures was designed to obtain as complete removal as possible of excess seed virus, the presence of which had been found to obscure initial increases in the virus content of samples of tissue and liquid. Equal parts of a 10% (w/v) suspension of cells and seed virus filtrate were mixed at 37° for 1 hr. either in a rotating bottle or preferably, to avoid packing of cells, by a magnetic stirrer. This suspension was then centrifuged at low speed and the supernatant discarded. The deposit was resuspended in Tyrode solution, centrifuged and the supernatant discarded. The deposit was then resuspended in strain-specific immune serum, left for 5 min., centrifuged and the supernatant discarded. The deposit was again resuspended in Tyrode solution, centrifuged and the supernatant discarded. This washing procedure was repeated 4 or 5 times. The deposit was finally resuspended in culture medium to the original volume to give a 10% (w/v) concentration of cells. A cell count was made at this stage to check for losses during washing, for which adjustment was made.

Cultures were either made in multiples of 5 or 10 ml. volumes in 30 ml. screw-capped bottles, or one culture of up to 100 ml. in a 250 ml. bottle was used, with repeated withdrawal of samples. The air in the bottles was replaced

before incubation with a mixture of 95% (v/v) oxygen+5% (v/v) carbon dioxide. During incubation the bottles were slowly rotated at an angle of 10–20° from the horizontal. From the time of mixing the seed virus filtrate with the cell suspension, the work was done in a hot room at 37°. The filtrate, cell suspension, culture medium, immune serum and the washing liquids were brought to 37° before use.

Preparation of infective material and estimation of its virus content

Six different preparations have been made. The culture-liquid sample was collected from the supernatant fluid following sedimentation of the cells in a horizontal bench centrifuge. The deposit of cells was ground with sand or coarse glass powder, resuspended in M/25 phosphate buffer solution (pH 7.6), centrifuged and the supernatant collected as the phosphate extract. The deposit of ground cells was sometimes resuspended in buffer solution and titrated without further centrifugation. The whole culture was titrated in two ways, first by making dilutions of the intact cells, suspended in the culture liquid, and secondly by depositing the cells, grinding them and resuspending them in their supernatant culture liquid. Finally the deposited cells after grinding were resuspended in antiserum, washed 4 times by repeated centrifugation and resuspended in buffer solution. Tenfold dilution series are prepared in M/25 phosphate buffer solution (pH 7.6). Three or more usually four dilutions were inoculated intraperitoneally into groups of five unweaned mice, using a 0.03 ml. dose (Skinner, 1951). The 50% end-point dilution was calculated by the method of Reed & Muench (1938) and the virus content expressed in ID₅₀/g. tissue or /ml. liquid. Calculation by Pizzi's method (1950) of the standard errors of the results of these titrations gave values of the order of ± 0.35 . In comparing the results of individual titrations, therefore, the smallest significant difference was unlikely to be less than 1 log unit. In all experiments to be described the necessary titration inoculations were made immediately after the collection and preparation of a sample had been completed. The interval between sampling by one worker was, because of this, never reduced to less than 15 min.

RESULTS

Preliminary investigations with fragments of tissue exposed to virus before incubation had shown that, following an initial drop in infectivity, the virus content of the tissue began to increase *c.* 3 hr. after incubation. The virus titre continued to rise for the next 1–3 hr., presumably by multiplication of virus in the cells initially infected. It seemed probable that by release of this virus, other susceptible cells became infected and by a series of such steps the maximum infectivity of the culture was reached between 12 and 24 hr. (Henderson, 1953*a, b*). A similar curve of multiplication was obtained when the culture of suspended tissue fragments was inoculated with the seed virus at the beginning of incubation, except that the initial decrease and increase in infectivity occurred about 1 hr. later. In all these experiments the presence

of the unutilized excess of seed virus obscured the first few hours of the growth curve until sufficient production of new virus had occurred to exceed this high basal value. The use of strain-specific immune serum in cell suspension cultures as described under culture methods became the standard way of dealing with this problem. The observations which led to the adoption of this method were made, however, by using tissue fragments.

Addition of immune serum to the culture medium. The addition of immune serum at 0 hr. (Henderson, 1953*a*) or at 30 min. produced a parallel infectivity curve about 2 log units lower than that shown by control cultures without serum. When the serum was added at 2–5 hr. there was, at first, a decrease in the infectivity of the tissue, but the value subsequently achieved was the same as that in the control culture (Fig. 1). From this it was concluded that by 2 hr. the virus concerned in multiplication could not be neutralized by immune serum, although excess seed virus was neutralized. Up to 30 min., however, the adsorption or the cellular penetration of the virus was incomplete and some could still be reached by the serum. These results with this culture system were very similar to those reported by Henle & Henle (1949) for influenza virus in the embryonated hen egg.

In the cell suspension culture method 1 hr. was allowed to elapse before the cells were separated from the seed virus filtrate and added to the immune serum; this manipulation and the subsequent washing took about 1 hr. This first hour for adsorption or penetration is thus the maximum permissible if the various procedures are to be completed before the occurrence of the first increase in infectivity.

Counts of trypsinized cell suspensions

The epithelial cells present in these cell suspensions can be divided into large cells and small cells. The large cells are flattened, irregular in shape with much cytoplasm, and intercellular processes may be visible on the cell membrane. These and the more elongated keratinized cells are arbitrarily classified as 'prickle' cells. The small cells tend to be spherical, are very refractile with little but dense cytoplasm; these cells are classified as 'basal' cells.

The number of cells of each type present depends on which layers are included in the strips of tongue epithelium. The proportion of basal cells may be as high as 70–80%. These cells survived the various centrifugation and washing procedures of the culture technique much better than the prickle cells which did not appear to be of much significance in the multiplication of the virus. A typical series of cell counts made during an experiment is shown in Table 1.

A preparation consisting almost entirely of viable basal cells was prepared by incubating a cell suspension overnight in bovine amniotic fluid followed by incubation with trypsin for 1 hr. It was found that few prickle cells survived the overnight incubation and thus only fully viable basal cells were left, the dead cells being digested by the trypsin.

Table 1. *Suspended cattle-tongue epithelial cell cultures of the virus of foot-and-mouth disease*

Differential counts after staining with eosin (% of total cells)

Sample	Prickle cells		Basal cells		Total basal cells	Total cell count (log no./ml.)
			Stained	Unstained	Stained	Unstained		
Original 1/10 cell suspension			4.4	8.0	7.1	80.3	87.4	6.65
After washing:								
(a) Virus culture								
10 ID 50/cell								
2 hr.			15.5	0.1	20.3	61.1	81.4	6.79
7 hr.			20.0	1.0	44.8	34.2	79.0	6.83
9 hr.			19.6	0.1	58.4	21.7	80.1	6.77
27 hr.			20.0	0	80.0	0	80.1	6.82
(b) Control								
No virus								
2 hr.			16.1	1.2	28.9	53.6	82.5	6.77
7 hr.			21.4	1.1	38.0	39.2	77.2	6.62
9 hr.			30.4	0.7	37.1	31.7	68.6	6.59
27 hr.			29.1	0.5	55.2	15.1	70.3	6.58

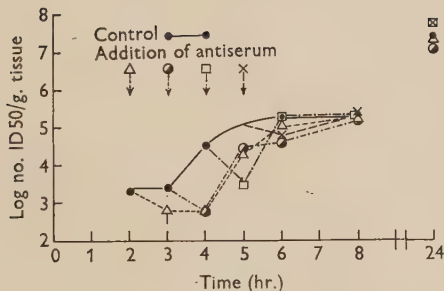


Fig. 1

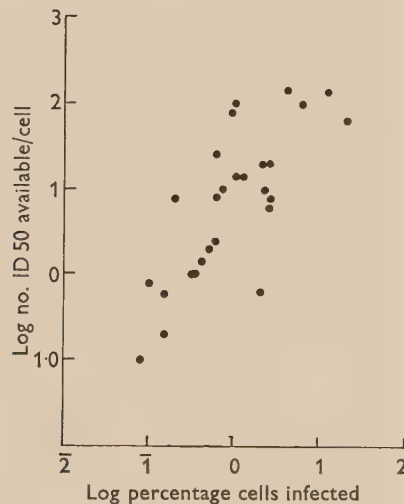


Fig. 2

Fig. 1. Effect of the addition of antiserum to cultures of the virus of foot-and-mouth disease. The infectivity is shown of the phosphate extract of the cells of the control culture and of four cultures to which serum was added at the times indicated. The 24 hr. titres are shown in the top right-hand corner.

Fig. 2. Relationship observed in 27 cultures between the amount of foot-and-mouth disease virus available to each cell and the number of cells that became infected.

The adsorption period

Earlier work with fragments of tissue and without the use of antiserum to neutralize excess seed virus failed to show any evidence of specific adsorption of the virus by the tissue in which multiplication subsequently took place. It could only be shown that the addition of tissue to a virus suspension resulted in the tissue acquiring a virus content, the amount of which was directly proportional to the amount of virus available but was little dependent on time and temperature (4° and 37°). When such tissue was added to a virus-free liquid, the liquid immediately acquired a virus content and the virus content of repeated washings did not greatly diminish; an equilibrium, in fact, was quickly established between tissue and liquid.

The use of antiserum and a suspension of separate cells provided new opportunities for studying the process of adsorption. Following exposure of a known number of epithelial cells to a known amount of virus and removal of excess seed virus, the cells could either be ground and a phosphate extract titrated (which provided a quantitative estimate of the infective virus content) or a titration of the cells, intact and undamaged, could be made. The latter method did not provide an estimate of the virus content but it gave an estimate of the number of infected cells. After mouse-inoculation virus multiplication proceeds in the intact infected cell with subsequent release of virus which then infects the mouse. The end-point dilution is that in which the percentage of negative samples is 50, i.e. e^{-m} of the Poisson distribution = 0.5, and m the mean number of infectious units or, in this case, infected cells = 0.7. The number of infected cells, therefore, was 0.7 times the reciprocal of the 50% end-point dilution.

The results of titrations of the phosphate extract of cells after the use of the antiserum and subsequent washing technique showed little or no infective virus to be present. Inoculation of dilutions of the intact cells into mice, however, showed that adsorption of virus had taken place (Table 2). The question of whether this adsorbed virus then became non-infective will be examined in discussing the latent period of the infectivity curve.

The data in Table 2 show that, judging by the number of infected cells, adsorption appeared to be complete within *c.* 15–20 min. There is an indication that the rate and frequency of infection increased with an increase in the amount of virus available. During the first hour of exposure far fewer cells adsorbed virus and became infected at 4° than at 37°, but when the period of exposure was prolonged for 4–6 hr. the degree of infection became as high as that reached at 37°.

Apart from these experiments in which some variation was introduced, the standard period of exposure was 1 hr. The data from twenty-seven experiments show a positive correlation between the amount of virus available and the percentage of cells that became infected (Fig. 2); these percentages are of the total number of cells. The results of differential cell counts, (Table 1) suggest that it was the basal cell that was involved in multiplication of the virus. The initial proportion of viable basal cells was usually 50–80% of the total. The

Table 2. *Exposure of known numbers of cattle-tongue epithelial cells to known amounts of foot-and-mouth disease virus for varying periods at 37° and 4° followed by removal of excess virus by the use of antiserum*

The number of cells becoming infected was determined by dilution of the cells, intact and undamaged, and inoculation of unweaned mice.

Cells (log no./ml.)	Virus (log ID 50/ml.)	ID 50/cell	Period of exposure (min.)	Infected cells (log no./ml.)	
				37°	4°
6.6	6.4	0.6	5	2.6	—
			15	2.9	—
			30	3.8	—
			45	3.6	—
			60	3.8	—
6.8	7.2	2.5	5	3.9	2.0
			15	4.6	3.0
			30	4.8	3.0
			45	4.8	3.0
			60	4.6	3.0
6.5	7.4	8.0	15	3.9	3.0
			30	3.9	2.9
			45	4.0	2.9
			60	3.8	3.0
			120	3.9	2.9
			240	6.4*	4.1
			360	7.2*	3.9
6.7	6.6	0.8	5	2.6	—
			15	3.2	—
			30	3.0	—
			45	3.8	—
			60	3.8	—
	7.6	8.0	5	3.8	—
			15	4.0	—
			30	4.4	—
			45	4.3	—
			60	4.5	—
	8.6	80.0	5	4.9	—
			15	4.9	—
			30	(3.8)	—
			45	4.8	—
			60	4.6	—

* Increase in infectivity due to multiplication.

percentage of viable basal cells that became infected would, therefore, never have been more than twice the value shown in Fig. 2. As the proportion of cells that became infected in this system was relatively low, the absolute values are little affected and relative differences remain the same.

Titration of the seed virus filtrate during the period allowed for adsorption provided little information because of the relative insensitivity of the test except when the amount of virus available was small. Any decrease in infectivity represented more than specific adsorption by susceptible cells, since virus 'acquired' by the cells and virus adsorbed by non-viable cells is included.

Fig. 3 shows the results obtained with three concentrations of seed virus, and it includes the amount of infectivity lost by thermal inactivation during the critical period of the experiment. It will be seen that with a low concentration of virus a decrease in infectivity might continue for 1–2 hr., this being consistent with the lower probability of collision between virus particle and cell compared with the experiments which provided the data in Table 2.

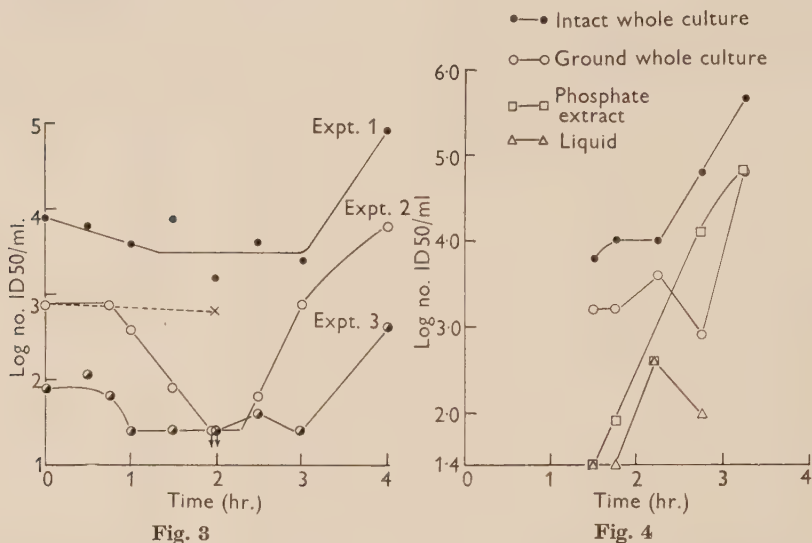


Fig. 3. The virus content of the liquid portion of mixtures of virus and cells incubated at 37°. The low concentrations of virus used allowed the drop in infectivity due to adsorption to be detected. The dotted line shows the drop in infectivity due to thermal inactivation. The symbol ♀ denotes that no virus was detected in the undiluted preparation.

Fig. 4. The infectivity of various components of a cell suspension culture of foot-and-mouth disease virus during and immediately following the latent period.

The latent period

In identifying portions of the infectivity curve, we define the latent period as beginning with the initial decrease in infectivity when the cells are exposed to virus and ending with the increase in infectivity observed in phosphate extracts of cells which is associated with the appearance of new virus. By the methods employed, these limits cannot be exactly determined for the whole culture, far less for an individual cell + virus unit; the definition is therefore somewhat arbitrary.

In the earlier experiments there was a comparatively high virus content associated with all the components of the culture from the time it was started. An increase above this was, however, detectable after *c.* 3–4 hr. From the results of the present experiments with the use of antiserum it is evident that the high initial virus content was due to excess seed virus and that the true beginning of the increase in infectivity was not detected because of the high threshold that had to be surmounted.

In a series of four comparable experiments with ID50:cell ratios of 4, 19,

13 and 8:1, respectively, samples were collected every 15 min. starting at 1.75 hr. Phosphate extracts of the cells were prepared and titrated and dilutions of the whole culture, i.e. the intact cells in the culture medium, were also prepared and inoculated into mice. The first increase in infectivity was detected at 2.5, 2.5, 2.25 and 2.25 hr. in the phosphate extract, and at 3, 3, 3.25 and 2.5 hr., respectively, in the whole culture. The two sets of times differed because titration of the phosphate extract detected any increase in infective virus in or attached to the cell whereas titration of the whole culture provided an estimate of the number of infected cells, and no increase in titre was observed so long as any increase in infective virus remained intracellular. An increase in infectivity was observed only when virus was released into the culture liquid and the amount exceeded the threshold imposed by the initial number of infected cells. The time periods cited are from the time of adding the seed virus to the cell suspension; the adsorption period was thus included. Table 2 shows that a substantial number of cells had adsorbed virus within 5 min.; it would presumably be these cells which would be associated with the first increase in virus; 2.25–2.5 hr. is therefore the approximate length of the latent period.

Infectivity of the virus during the latent period

Because of the well-recognized eclipse phase or period of non-infectivity which occurs with the multiplication of bacteriophages and with a number of animal viruses, special attention was paid to this part of the latent period in the present experiments. In searching for evidence in a cell-suspension culture the following preparations were tested: (a) the culture liquid; (b) the phosphate extract of the ground cells; (c) the debris of the ground cells used in the preparation of (b); (d) the intact whole culture, i.e. the undamaged cells suspended in the culture liquid; (e) the ground whole culture, i.e. the cells ground and the debris resuspended in the culture liquid. A typical result is shown in Fig. 4. Little or no infective virus was detected in the culture liquid or in the phosphate extract of ground cells, whereas inoculation of mice with intact cells demonstrated the presence of virus which, if non-infective at the time of sampling, became infective with consequent paralysis and death of the mouse. Infective virus was also detectable in the debris of the cells used to prepare the phosphate extract.

The virus was not released during preparation of the extract in the way that it is readily extracted during the period of increasing infectivity. This demonstration of infectivity may be due to virus adsorbed by insusceptible cells not being neutralized by the antiserum, to the presence of infected cells which escaped disruption during the process of grinding, or to a proportion of virus that remained infective and intimately associated with cellular material.

These points were examined experimentally with the following results.

(1) Possibility of incomplete neutralization of non-specifically adsorbed virus. A cell suspension was prepared and a portion heated for 10 min. at 65°. The heated cells were stainable with eosin. Heated and unheated cells were separately mixed with virus for 1 hr. at 37°, washed in the routine manner,

using antiserum, and resuspended in culture medium. At 2 hr. titrations were made of the intact cells. No infectivity was detected in the heated cell-preparation, whereas the other contained $10^{2.6}$ infected cells/ml.

(2) Possibility of infected cells surviving grinding. Microscopical examination of smears of cellular debris showed that grinding could be carried to a stage where the presence of an undamaged cell was sufficiently rare to be discounted as a possible explanation. Other methods of disruption were tried but none replaced grinding for these cells. For example, rapid freezing in solid CO_2 + ethanol followed by thawing in a water-bath at 37° repeated 8 times, killed the cells but did not disrupt them. Slow freezing and slow thawing was no more successful. Severe changes in osmotic pressure caused the cells to be stainable with eosin but they remained intact.

(3) Possibility of intimate association of infective virus with cellular material. It was confirmed on a number of occasions that although a phosphate extract of cells sampled during the latent period contained little or no detectable virus, a comparatively high degree of infectivity was caused by the debris of the cells used to prepare these extracts. A similar degree of infectivity was found in the ground whole-culture preparations which were obviously associated with the cellular debris, in view of the low titre of parallel samples of the culture liquid. As the virus in this debris appeared to be firmly bound an attempt was made to determine whether it was exposed to the neutralizing action of antiserum. The data for the latent period in a number of experiments is given in Table 3; in each case the culture liquid contained little virus. The cellular debris had an infectivity titre of the same order as the number of infected cells. Little or none of this infective virus was extracted by $\text{M}/25$ phosphate buffer solution (pH 7.6) and at least 10% of it was not neutralized by antiserum.

It can be concluded, therefore, that during the latent period the association between the virus and the cell is one of close union and that unless all the fractions and components of the system are examined, it might be thought that the absence of virus from tissue extracts denoted that virus was non-infective during this period.

Reference to Fig. 6, in which the effect is illustrated of varying the initial ID50:cell ratio, shows that when all the cells in the culture were not infected by the original exposure to virus, there was a characteristic latent period associated with each subsequent batch of cells to become infected.

The period of increasing infectivity

At the end of the latent period an increase in infectivity was observed in all the components of the culture. This increase was at first associated with the cells or in the preparations derived from them. The increase in the phosphate extract, for which there is most data, has been used to define the end of the latent period. An increase in the infectivity of the culture liquid or in the phosphate extract means that more virus has been released from the cells or is extractable from the disrupted cells.

The increases of infectivity in the phosphate extract, the ground whole

culture and in the debris treated with antiserum appeared to occur simultaneously. An increase in the infectivity of the intact whole culture and of the culture liquid may not be detected until about 30 min. later. The result of an experiment in which the data for all these components were obtained is shown in Fig. 5. It will be seen that there was more virus in each of the intact whole-culture samples than in the corresponding samples of the culture liquid, in the

Table 3. *Virus infectivity during the latent period of the multiplication of foot-and-mouth disease virus in suspended cell cultures*

Demonstration in the debris of ground cells of infectivity not extracted with phosphate buffer solution and not wholly neutralized by antiserum.

Initial ID 50/cell	Time (hr.)	Infected cells (log no./ml.)	Culture liquid (log ID 50/ml.)	Phosphate ext. (log ID 50/ml.)	Cellular debris (log ID 50/ml.)	Debris + antiserum (log ID 50/ml.)
2	0.50	3.3	—	0	2.9	—
63	1.50	3.6	1.4	0	3.2	2.9
	1.75	3.8	0	1.9	3.2	4.0
	2.25	3.8	2.6	2.6	3.6	2.2
10	2.25	4.4	—	—	2.7	3.2
	2.50	4.0	—	—	3.2	2.4
30	2.25	4.8	0	1.9	4.6	2.6
100	0.50	4.7	0	0	3.6	3.6
	1.00	3.8	—	—	3.4	3.6
	1.50	3.9	0	1.4	3.4	3.0
40	0.50	3.8	—	—	4.6	2.8
	1.00	4.4	—	—	3.9	3.0
	1.50	4.4	1.6	1.4	4.8	2.9
	2.00	4.2	(3.2)*	1.4	4.5	2.8
10	0.50	3.0	—	0	3.2	—
	1.00	3.4	—	1.4	2.8	—
	1.50	3.8	—	0	2.9	—
Mean		4.2	1.8	1.7	4.1	3.3

* Excluded in calculating the mean. The value for the 2.50 hr. sample was 1.4.

phosphate extract or in their sums. The few values available for the ground whole-culture samples, however, are similar to those for the whole culture. The greater than thousandfold increase in the infectivity of the whole culture cannot be accounted for by a corresponding increase in the number of infected cells as the initial ID 50:cell ratio of 40 would be expected to infect all the susceptible cells and the total cell count was only $10^{6.6}$ /ml., as compared with the plateau of $10^{7.9}$ ID 50/ml. reached by the whole culture. It seems probable that much of the new virus was adsorbed by dead cells and cellular debris. All this would be present in the ground whole-culture preparation but would be centrifuged out of the culture liquid and the phosphate extract samples. The results of treating the deposit with antiserum show that all but a small proportion of this adsorbed virus was neutralized.

It is more difficult to account for the increase in the infectivity of the deposit treated with antiserum. It was concluded that during the latent

period this unneutralized virus was closely associated with infected cells. Had it been reasonable to suppose that more cells had become infected following the first release of virus it might have been assumed that this was reflected in this increase in infectivity. The coincidence in the occurrence of this increase with the rise in infectivity of the other cellular components suggests that it might be related to cells becoming producers of virus. This same pattern has

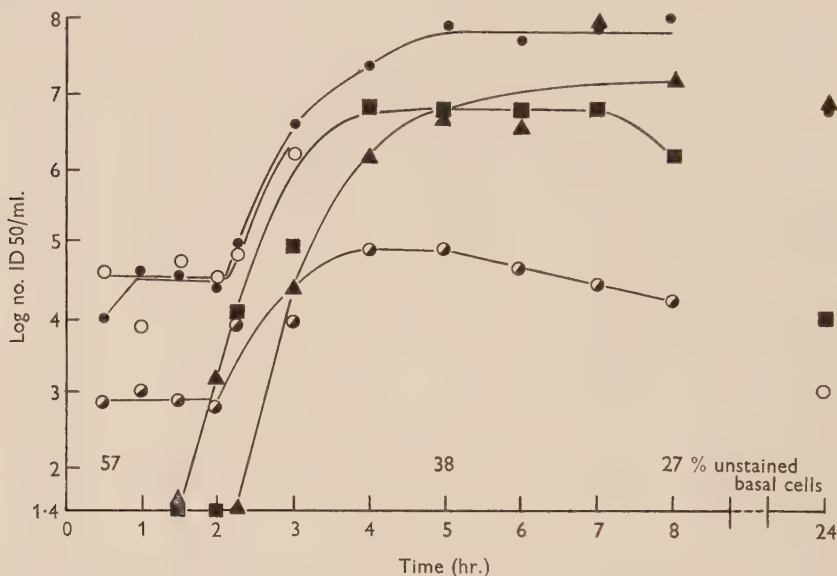


Fig. 5. The period of increasing infectivity. The preparations examined were the culture liquid (▲-▲), the phosphate extract (■-■), the intact whole culture (●-●), the ground whole culture (○-○) and the ground whole culture treated with antiserum (◐-◐).

been observed in two other experiments for which complete data are available. In these, the ground whole-culture samples were titrated hourly up to 6 hr.; the results were similar to those for the intact whole culture.

There was some variation in the duration of the period of the increase in infectivity and, when judged by the virus content of the phosphate extract, its magnitude increased with an increase in the number of infected cells. The increase in infectivity was, however, comparatively constant when the number of infected cells and the peak of the first increase in infectivity attained by the whole culture were related to a unit period of 15 min. The relevant data from fourteen experiments are given in Table 4.

The result of exposing the cells to different concentrations of seed virus is shown in Fig. 6; ID₅₀:cell ratios of 10:1, 1:1 and 0.1:1 were chosen. The numbers of cells which became infected were directly proportional to these values, with a tenfold difference between each. There was also a directly proportional tenfold difference between the peaks of infectivity reached following the initial infections. The three curves may be interpreted as showing that 10 ID₅₀/cell were sufficient for primary infection of all susceptible cells,

but that 1 and 0.1 ID₅₀/cell were insufficient. With 1 ID₅₀/cell all became infected after the first release of virus, whereas it was not until there had been a release of virus from the second batch of cells which became infected that all were involved in the culture infected with 0.1 ID₅₀/cell.

Table 4. *The increase in infectivity of suspended cell cultures of the virus of foot-and-mouth disease related to the number of infected cells, the peak of the first increase in infectivity attained by the whole culture and reduced to a unit period of 15 min.*

Infected cells (log no./ml.) (a)	Peak of infectivity (log ID ₅₀ /ml.) (b)	Duration of 1st period of increase (hr.) (c)	$\frac{(b)-(a)}{(c) \div \frac{1}{2}}$ (log ID ₅₀ /cell/15 min.)
3.05	6.0	1.50	2.2
3.65	6.9	2.00	2.4
3.85	7.0	1.00	2.6
3.85	7.2	2.00	2.5
4.05	7.2	1.00	2.6
4.05	7.2	1.75	2.3
4.25	7.8	1.75	2.7
4.25	7.8	2.25	2.6
4.45	7.9	3.00	2.4
4.75	7.9	1.00	2.6
4.85	7.0	0.75	1.7
4.85	7.9	1.75	2.2
5.05	8.4	1.25	2.7
5.65	8.0	0.50	2.1

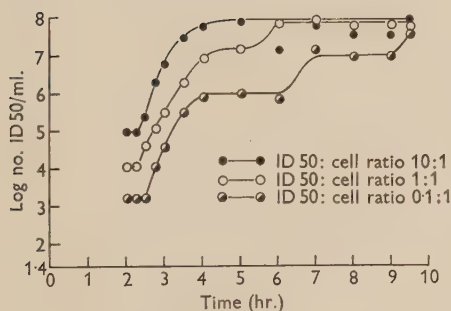


Fig. 6

Fig. 6. The infectivity of the intact whole cultures in which three virus concentrations were used.

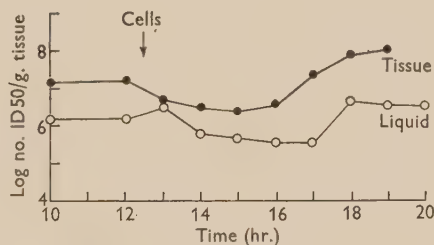


Fig. 7

Fig. 7. The effect of adding susceptible cells to a culture during the period of the plateau of maximum infectivity.

A feature of these suspended cell cultures in which there was complete initial infection was that the degree of infectivity ultimately reached was maintained at a relatively constant value for about 4–6 hr. The addition of susceptible cells to the culture during this period resulted, after the appropriate latent period, in a further increase in infectivity (see Fig. 7).

Period of decreasing infectivity

The plateau observed after the period of increase was usually maintained until between *c.* 10 and 12 hr.; thereafter there was a decrease in the infectivity of all the components of the culture. This decrease occurred more rapidly in the tissue than in the liquid. By 24 hr. the virus content of the culture was essentially that of the liquid, its degree of infectivity being about 1 log unit lower than at 12 hr. Cell counts made at 27 hr. (Table 1) showed that no viable cells remained. The decrease in infectivity appeared to be due to death of virus-producing cells, combined with thermal inactivation of the virus.

DISCUSSION

These experiments have been concerned almost entirely with the first 8 hr. of incubation of this culture system; no data are presented on any characteristic of the virus other than infectivity. The complement-fixing activity and the immunizing activity of the antigen were not detectable until the virus content of the culture exceeded 10^5 or 10^6 ID₅₀/ml. Ubertyni *et al.* (1956), who studied the development of these three characteristics in cultures of cattle-tongue tissue fragments, only detected infectivity up to 6 or 12 hr. The method here used to determine the number of infected cells by titration of the intact whole culture during the adsorption and latent periods was based on the assumption that, since the phosphate extract was practically non-infective, 'maturation' of non-infective virus had to occur within undamaged cells after inoculation into the mouse. The fact that the most recent experiments have shown that a similar end-point is found when the ground whole culture is titrated does not invalidate the method, but it seems unnecessary to assume that any maturation is required or that cells need survive within the mouse. It must be noted that only about 1% of the total cells, as determined by the titration method, becomes infected, whereas the number of viable basal cells is usually between 70 and 80%. It has been concluded, therefore, that the titration method may be accepted for the estimation of relative numbers, but not necessarily for absolute numbers, of infected cells. When, however, simultaneous titrations have been done, on a few occasions, by the plaque-counting method (Sellers, 1955) the same result has been obtained.

The production of infection by inoculation of mice with preparations of ground cells collected during the latent period provides evidence that much virus, apparently specifically adsorbed, remains infective during this period. Ackermann, Ishida & Maassab (1955) described the occurrence of bound infectious virus in chick chorioallantoic membrane infected with influenza virus. They concluded that this bound virus did not function in the initiation of virus increase. There is some similarity with our observations on the presence of virus closely associated with the cells during the latent period, but we have no evidence that this virus should be disregarded in considering the subsequent increase in infectivity. If inoculation of a number of non-infective units or subunits of virus may be followed by their organization and

the formation of an infective unit within the mouse, it must be recognized that this might be equally true at any time for any infective virus.

It is apparent from the literature that the search for infective virus during the latent period has not always been sufficiently exhaustive. The failure to recover infective virus from the supernatant fluid of a centrifuged suspension of ground tissue has, on occasion, been accepted as evidence of the occurrence of an eclipse phase. There is no doubt, however, that a difference existed in these experiments in the facility with which virus could be extracted or eluted from the cellular material in the later stages of its multiplication as compared with that during the latent period.

Attempts have been made with our culture system to establish the form of the differential growth curve in which the yield of virus/unit time interval is determined. The sedimentation of the cells by centrifugation for removal of the medium and during the subsequent washing procedures before new medium was added eventually killed so many cells that to do this with the necessary frequency became impracticable. The results of performing this type of experiment on monolayer cultures of bovine amniotic ectoderm (Pay, 1956) show that such cells infected with the virus of foot-and-mouth disease may release virus at a constant rate for 2-3 days. In monolayer cultures of bovine-tongue epithelial cells (Pay, unpublished) the same constant release has also been observed with one type of cell-growth whereas another type of cell-growth undergoes fatal cytopathogenic changes within 24 hr. The fact that virus production ceased so much earlier in suspended-cell culture was undoubtedly due to the inability of many of these cells to remain viable in a liquid medium, whether or not they were infected, and because the remainder of the cells died, probably as a result of specific pathogenic changes.

Table 5. *Increase in infectivity/15 min. in suspended cell cultures of the virus of foot-and-mouth disease in which the initial concentration of virus was 10, 1 and 0.1 ID 50/cell*

Time at end of 15 min. period (hr.)	Increase in ID 50/ml./15 min.		
	Culture 10	Culture 1	Culture 0.1
2.25	0	0	0
2.50	150,000	28,000	0
2.75	1,750,000	80,000	8,400
3.00	4,300,000	200,000	25,000
3.25	11,700,000	580,000	85,000
3.50	14,000,000	1,300,000	200,000
3.75	18,000,000	2,800,000	240,000
4.00	13,000,000	4,000,000	240,000
4.25	17,000,000	3,000,000	200,000
4.50	0	3,000,000	0
4.75	0	0	0
Mean of last 4 values	$10^{7.2}$	$10^{6.5}$	$10^{5.3}$
Latent period titre	10^{-5}	$10^{-4.1}$	$10^{-3.2}$
Infected cells/ml.	$10^{4.85}$	$10^{3.9}$	$10^{3.05}$
ID 50/cell/15 min.	$10^{3.4}$	$10^{3.6}$	$10^{2.3}$

Work with monolayer cultures suggests that the results of the present experiments should be interpreted on the assumption that an infected cell at the end of its latent period becomes a producer of virus, releasing it at a constant rate. Analysis of the data of the experiment illustrated in Fig. 6 shows that this hypothesis is consistent with the observations. The increase in infectivity during each 15 min. from the end of the latent period to the first peak (Table 5) suggests that during the last four 15 min. periods a constant rate of virus production had been reached in each culture, i.e. that all infected cells were then virus producers. In the two cultures with incomplete initial infection, the infectivity would in part be due to the contribution of the number of cells in the second batch which became infected, but it can be disregarded since the maximum number available for subsequent infection was such a small proportion of the degree of infectivity attained. In the culture with an initial ID 50:cell ratio of 10:1, $10^{4.85}$ infected cells/ml. had an average production of $10^{7.2}$ ID 50/ml. during each of the later 15 min. periods, i.e. $10^{2.4}$ ID 50/cell/15 min. The corresponding values are $10^{2.6}$ and $10^{2.3}$ for the 1:1 and 0.1:1 cultures, respectively. If these figures be assumed to represent the rate of virus production by an infected cell, the number of virus-producers in action during the earlier 15 min. periods may be calculated from the total amount of virus produced during each period; the results of these calculations are given in Table 6. A similar analysis of the data of the experiment illustrated in Fig. 5 gives a virus production figure of $10^{2.9}$ ID 50/cell/15 min.

Table 6. *Percentage numbers of infected cells which became virus producers during the period of increasing infectivity as calculated from the data in Table 5*

Time at end of 15 min. period (hr.)	Calculated proportion of infected cells which became virus producers (%)		
	Culture 10	Culture 1	Culture 0.1
2.25	0	0	0
2.50	1	1	0
2.75	10	2	4
3.00	24	6	11
3.25	66	16	38
3.50	100	36	100
3.75	—	100	—

The values in Table 4, in which the total increase in infectivity is related to a 15 min. period, are underestimates of the mean virus production/cell. They would be correct only if all the cells were producing virus for the duration of the period of increase. No account is taken of the hypothesis that the number of cells which produce virus gradually increases throughout the first 60–75 min. Comparison of values obtained for 15 min. periods from the same data by the two methods of calculation suggest that the values shown in Table 4 are underestimates by about 0.4 log unit; the mean corrected value for these data is $10^{2.9}$. The conclusion reached is that the rate of virus production/cell/15 min. is probably of the order of 10^2 to 10^3 ID 50.

Assessment of the significance of the plateau at the end of the period of increase in infectivity is complicated by the fact that at this high value an absolute increase in amount of virus must be very considerable before it can be detected. In the experiment illustrated in Fig. 6 a peak of 10^8 ID₅₀/ml. was reached in the 10:1 culture. The estimated production attained by this time was estimated to be $10^{7.2}$ ID₅₀/15 min. This rate of production would have to continue for 1.75 hr. before a twofold increase had occurred, an amount not detectable by the titration method used. It is hardly possible, therefore, to conclude whether the plateau is apparent or real. The influence of thermal inactivation must be considered; a plateau would be maintained so long as thermal inactivation balanced new production. Death of infected cells would lead to a disturbance of this balance and result in the decrease of infectivity observed later. In the experiment illustrated in Fig. 5 a decrease occurred in the infectivity of the ground cells treated with antiserum and in the number of viable basal cells, while the plateau was still present. This suggests that death of infected cells might have taken place before any decrease in infectivity was large enough to be observed.

The conclusions reached are that in the described culture system the pattern of multiplication of the virus of foot-and-mouth disease was that adsorption of the virus by the cells occurred rapidly and, except with low concentrations of virus, was complete in 15–30 min. The virus then became closely associated with the cells and was not neutralized by antiserum, nor readily extractable, but retained infectivity. This state persisted during a latent period of about 2.5 hr. At the end of this period some of the epithelial cells produced new virus at a steady rate of about 10^2 to 10^3 ID₅₀/15 min. and all the infected cells were producing within *c.* 1 hr. This production continued so long as these cells survived; thereafter the infectivity of the culture declined, ultimately, because of thermal inactivation of virus. In this culture system this decline started after *c.* 12 hr.

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The Mutation of *Corynebacterium pyogenes* to *Corynebacterium haemolyticum*

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SUMMARY: *Corynebacterium pyogenes* has been observed to give rise to mutants which are indistinguishable from *Corynebacterium haemolyticum*. *C. pyogenes* ferments lactose and xylose (Xyl+), elaborates a soluble haemolysin and/or a proteolytic enzyme (H⁺), and its cell walls contain glucose units in addition to certain other components. On horse blood agar *C. pyogenes* forms small colonies (s) surrounded by large zones of haemolysis. *C. haemolyticum* and the mutant derived from *C. pyogenes* ferment lactose but not xylose, produce no soluble haemolysin, and glucose cannot be detected in hydrolysates of their cell walls. The change in the basal structure of the cell wall is accompanied by a lack of immunochemical cross-reaction between the wild type and the mutant or *C. haemolyticum*. On horse blood agar both *C. haemolyticum* and the mutant produce relatively large colonies (L) surrounded by narrow bands of haemolysis. The possibility that a single mutation involving cell-wall structure may account for the apparent change from Xyl⁺H⁺ to Xyl⁻H⁻ is discussed. It is suggested that neither *C. pyogenes* nor *C. haemolyticum* is a corynebacterium, and that taxonomically both organisms belong to the genus *Streptococcus*.

Corynebacterium pyogenes has long been known to be unique among the corynebacteria which cause disease in man in that it ferments lactose, does not form metachromatic granules and produces a soluble haemolysin (Brown & Orcutt, 1920; Lovell, 1937; Topley and Wilson's *Principles*, 1955). Another corynebacterium, *C. haemolyticum*, which shares some of these properties, was described by MacLean, Liebow & Rosenberg (1946) who suggested that it resembled *C. ovis* and *C. pyogenes*. Evidence to be presented here indicates that *C. haemolyticum* is a mutant of *C. pyogenes*, and that as already suggested (Cummins & Harris, 1956) both of these organisms belong to the genus *Streptococcus* rather than the genus *Corynebacterium*.

METHODS

Sources of strains. Several strains of *Corynebacterium pyogenes* and a few strains of *C. haemolyticum* were isolated between 1945 and 1949 from the throats of patients suffering with acute pharyngitis, from urethral exudates and from cutaneous lesions. This part of the work was carried out at the

406th Medical General Laboratory, U.S. Army, Tokyo, Japan. Additional strains of *C. pyogenes* were kindly supplied by Professor R. Lovell, Dr I. A. Merchant, Dr F. Potoshka (Charles University, Prague), and Miss Dorothy Ballard (see Lovell, 1937, 1941; Merchant, 1935; Ballard, Upsher & Seely, 1947). A culture of strain NCTC 5224 maintained by the National Collection of Type Cultures (Colindale, London, N.W. 7, England) was obtained from Dr S. T. Cowan. The type strain of *C. haemolyticum*, 53-W-1, and the strain 53-W-2, were supplied by Dr A. A. Liebow, together with cultures of *C. ulcerans* (New York State Department of Health, Albany, N.Y., U.S.A.). Strains of *C. diphtheriae gravis*, *mitis*, *intermedius* and *C. xerosis* were provided by the United States Army Medical Department Research and Graduate School, Army Medical Centre, Washington, D.C. Several cultures of *C. ovis* were sent to us by Professor H. R. Carne (Carne, 1939). The biochemical patterns of these various strains are shown in Table 1. Information relative to the cell-wall components of representative corynebacteria and streptococci are given in Table 2.

Culture media

Basal medium for biochemical reactions. The proteolytic activity of *Corynebacterium pyogenes* excludes the use of Hiss's serum water as a basal medium for carrying out the classical fermentation tests. Further, most peptones which are commercially available inhibit the growth of this organism in concentrations of 0.5–1% (w/v) unless they are first treated with charcoal. Proteose-peptone no. 3 (Difco) permits suitable growth from large inocula when used in the concentration indicated in the following formula: proteose-peptone no. 3 (Difco) 2.5 g.; Na_2HPO_4 1.0 g.; NaCl 5.0 g.; distilled water 1 l. The pH value of this medium was adjusted to 7.4 and 8 ml. of a 0.2% (w/v) solution of phenol red was added. Volumes of 100 ml. were sterilized by autoclaving at 15 lb./sq.in. for 15 min.

With the exception of corn starch, all carbohydrates used (see Table 1) were of A.R. grade and were employed as sterile filtrates in a final concentration of 1% (w/v). Corn starch was used as a 0.4% (w/v) suspension autoclaved at 10 lb./sq.in. for 10 min. The complete medium (sterile basal medium + sterile carbohydrate solution) in each case was autoclaved at 7 lb./sq.in. for 10 min., and aseptically tubed in 2 ml. amounts in tubes of small diameter. For fermentation tests under anaerobic conditions, 0.1% (w/v) sodium thioglycollate was incorporated in the basal medium and, in this case, the completed and tubed medium received an overlay of sterile mineral oil 4 mm. in depth. For observation of gelatin hydrolysis, 8% (w/v) gelatin was used. Skim milk 10% (w/v) was prepared from the Difco dried product.

Medium for growth in bulk. Suspensions of organisms for disintegration were prepared from cultures grown in screw-capped bottles containing 1 l. of medium. The basal medium was infusion broth to which was added before sterilization 0.1% (w/v) sodium phosphate (Na_2HPO_4 , anhydrous). This medium was enriched before inoculation by the addition of sterile glucose + bicarbonate solution (10% (w/v) glucose + 10% (w/v) NaHCO_3 anhydrous) and

sterile horse serum, in the proportions of 2 ml. of each/100 ml. final medium. The bottles were inoculated with 4-5 ml. of overnight culture in the same medium, and incubated for a further 48 hr. Formaldehyde solution was then added to a final concentration of 0.5% HCHO and the culture allowed to stand at room temperature for 24 hr.

Cell-wall analysis. The preparation of cell-wall fractions, and the chromatographic examination of the products of hydrolysis, were done as previously described (Cummins & Harris, 1956). This involved disintegration of the organisms in a Mickle shaker, and treatment of the cell-wall fraction first with trypsin and ribonuclease and then with pepsin. The purified cell-wall material was hydrolysed, and the hydrolysates examined by two-dimensional paper chromatography. The amounts of the various components present were scored as + + +, + +, +, \pm , tr. or - depending on the size and intensity of the spots.

RESULTS

Cultural characteristics of Corynebacterium pyogenes and C. haemolyticum

One of the principal differences between *Corynebacterium pyogenes* and the variants of it described below is that of colony size, and this is indicated for the various strains under discussion by the letters *s* (=small colony) or *L* (=Large colony) in brackets after the strain number, e.g. 637 (*s*) 13081 (*L*), etc.

Strains 14-1-s (Lovell), 637-s and 13081-s of Corynebacterium pyogenes. Following 24 hr. of incubation on blood agar *Corynebacterium pyogenes* (*s*) strains formed tiny colonies (in contrast to large (*L*) colony forms to be discussed later) surrounded by large zones of haemolysis (2 to 3 times the diameter of the colony). For some strains growth was much enhanced when the CO₂ tension in the atmosphere was increased. The individual organisms were wedge-shaped, from 0.5 to 2 μ . in length, and were Gram-positive. When stained with methylene blue or toluidine blue metachromasia was not evident. Colonies on inspissated serum (Loeffler's slopes) produced marked pitting as they digested the coagulated protein. Skim milk was readily clotted and in a few days the clot was completely digested. All strains fermented lactose and xylose (see Table 1); all failed to show any catalase activity, even when the organisms were first disintegrated in the Mickle shaker. With sodium caseinate it could be shown that coagulation of casein and digestion of the coagulum were both dependent upon the presence of calcium ions (see also Brown & Orcutt, 1920). Cell-free preparations preserved with either 0.01% (w/v) thiomersalate or 0.5% (w/v) phenol caused the precipitation of casein and the subsequent digestion of the precipitate. The same preparations were haemolytic for human, guinea-pig, horse and rabbit erythrocytes. The haemolysin was stable to oxygen; there was no enhancement of its activity by reducing agents. Lovell (1941, 1944) demonstrated that antitoxin prepared against *C. pyogenes* toxin neutralized the toxin *in vivo* and the haemolysin *in vitro*. With antitoxin, provided by Professor Lovell, we were able to neutralize both the toxic action

for mice of our crude cell-free extracts as well as their haemolytic activity. Sera prepared against strains 637-*s* and 14-1-*s* showed cross-agglutination to within 50–100 % of their homologous titres; strain 13081-*s* antiserum was not prepared.

Strains 53-W-1 and 53-W-2 of *Corynebacterium haemolyticum*. On blood agar, after incubation for 24 hr., *Corynebacterium haemolyticum* grew as easily

Table 1. *Fermentation reactions given by various corynebacteria with the indicated sugars and alcohols*

All organisms gave negative reactions with mannitol, sorbitol, dulcitol, arabinose, rhamnose and raffinose.

	Glucose, fructose	Maltose, dextrin	Galactose	Sucrose	Starch	Lactose	Trehalose	Xylose	Inositol	Glycerol	Gelatin liquefaction
<i>Corynebacterium diphtheriae</i>											
gravis (53-A-7)	+	+	+	—	+	—	—	—	—	+	—
mitis (53-A-4)	+	+	+	—	—	—	—	—	—	+	—
intermedius (53-A-9)	+	+	+	—	—	—	—	—	—	—	—
mitis (53-A-15, 53-A-16)	+	+	+	+	—	—	—	—	—	+	—
<i>Corynebacterium ulcerans</i> (39164)	+	+	+	—	+	—	+	—	—	+	—
<i>Corynebacterium ovis</i> (CS1R-1, -2)	+	+	—	—	—	—	—	—	—	+	—
<i>Corynebacterium xerosis</i> (53-K-1)	+	—	+	+	—	—	—	—	—	—	—
<i>Streptococcus pyogenes</i> *	+	+		+	—	+	+	—	—	—	—
<i>Corynebacterium pyogenes</i>											
Small colony types											
Lovell (14-1- <i>s</i>)	+	+	+	—	—	+	—	+	—	—	+
(14-8- <i>s</i>)	+	+	+	—	+	+	—	+	—	—	+
Merchant (P-14- <i>s</i>)	+	+	+	—	+	+	—	+	—	—	+
The authors' (637- <i>s</i>)	+	+	+	—	+	+	—	+	—	—	+
(13081- <i>s</i>)	+	+	+	—	+	+	—	+	—	—	+
Large colony types											
variants from (<i>s</i>) strains											
(14-1- <i>L</i>)	+	+	+	—	—	+	—	—	+	—	—
(P-14- <i>L</i>)	+	+	+	—	—	+	—	—	+	—	—
(637- <i>L</i>)	+	+	+	—	—	+	—	—	+	—	—
(13081- <i>L</i>)	+	+	+	—	—	+	—	—	+	—	—
received as (<i>L</i>) strains											
Potoshka I, II, III <i>L</i>	+	+	+	+	—	+	—	—	—	—	—
Potoshka IV <i>L</i>	+	+	+	+	—	+	—	—	+	—	—
The authors' six strains <i>L</i>	+	+	+	—	—	+	—	—	+	—	—
The authors' two strains <i>L</i>	+	+	+	+	—	+	—	—	+	—	—
<i>Corynebacterium haemolyticum</i>											
Liebow 53/W/1	+	+	+	—	—	+	—	—	—	—	—
Liebow 53/W/2	+	+	+	—	—	+	—	—	—	—	—

* Reactions given in the 6th edition of *Bergey's Manual of Determinative Bacteriology* (1948).

visible colonies about twice the diameter of those of *C. pyogenes*, and surrounded by a narrow band of haemolysis just exceeding the diameter of the colony. The individual organisms were from 2 to 10 μ . in length, were always tapered and usually occurred in mats of several cells. They were Gram-positive

and when stained with metachromatic dyes did not show metachromasia. All strains fermented lactose and failed to ferment xylose. They showed feeble catalase activity in that the evolution of gas from a mixture of H_2O_2 and organisms was hardly discernible; fresh extracts obtained by breaking organisms up in a Mickle disintegrator, however, exhibited definite catalase activity. All strains coagulated milk but did not lyse the coagulum. Cell-free extracts showed neither haemolytic nor proteolytic activity. The organisms were capable of growing from small inocula on such ordinary laboratory media as Neopeptone broth. The growth of the organisms in matted clumps made them entirely unsuitable for the preparation of agglutinable suspensions.

Other ways in which various strains of *Corynebacterium haemolyticum*, *C. pyogenes* and other corynebacteria differ from one another are shown in Table 1.

The mutation of Corynebacterium pyogenes to *C. haemolyticum*

When *Corynebacterium pyogenes* strains 14-1-s, 637-s and 13081-s were repeatedly subcultured on blood agar there occasionally appeared a large colony variant, *L*, which possessed all of the cultural and biochemical properties of *C. haemolyticum*. Three such mutants were designated 14-1-*L*, 647-*L* and 13081-*L*. Over a period of several years no reverse mutation among them from *L* to *s* has been observed.

All the strains obtained from Dr Potoshka labelled *Corynebacterium pyogenes* were large colony strains, as were at least eight distinct isolates sent to us as reference cultures from various hospitals in Japan. All strains received from Professor Lovell and from Dr Merchant were small colony strains, or what we assume to be the wild type *C. pyogenes*. In all, four wild types, two from animal sources and two from human cases, were examined for evidence of the mutation from *s* to *L*. Four *L* variants, one from each of the wild types, were obtained. Selection was carried out on the basis of colony morphology alone. All four mutants gave identical fermentation and proteolytic reactions which were indistinguishable from those of *C. haemolyticum*. Since the *L* mutant grows fairly well on ordinary laboratory media, whereas the *s* does not, selection on such media may account for the preponderance of *L* variants in reference cultures from hospital laboratories.

Cell-wall composition of Corynebacterium pyogenes s and L

Determinations of the major components of the cell walls of eight strains of *Corynebacterium pyogenes s* and *L* were made. In all cases the main amino acid components were alanine, glutamic acid and lysine, and the characteristic sugar was rhamnose. This pattern of cell-wall composition closely resembles that previously reported for streptococci of different Lancefield groups, but it is decidedly unlike that of various corynebacteria (Cummins & Harris, 1956) where the characteristic amino acids of the cell wall are alanine, glutamic acid and diaminopimelic acid, and the characteristic sugars are arabinose and galactose (see Table 2).

The cell-wall composition of the wild type *Corynebacterium pyogenes*

differed from that of the *L* mutant. Glucose was regularly present in the wild type and absent from *C. haemolyticum* and the other *L* variants. Mannose was sometimes detected in the wild type but not in the mutant strains (Table 3).

Table 2. *Principal products of hydrolysis of cell-wall preparations from various corynebacteria, streptococci, Corynebacterium pyogenes and C. haemolyticum*

Data from Cummins & Harris, 1956.

	Arabinose	* Rhamnose	Alanine	Glutamic acid	Lysine	Diaminopimelic acid
Corynebacteria (<i>C. diphtheriae</i> , <i>C. hofmannii</i> , <i>C. xerosis</i> , <i>C. renale</i> , <i>C. ovis</i> , <i>C. ulcerans</i> , <i>C. equi</i> , <i>C. murium</i>)	+++	—	+++	+++	—	+++
Streptococci (representatives of groups A, B, C, D, E, F, G)	—	++	+++	+++	+++	—
<i>C. pyogenes</i> (6 strains)	—	++	+++	+++	+++	—
<i>C. haemolyticum</i> , strain 53/W/1	—	++	+++	+++	+++	—

* *C. murium* cell walls contained a small amount of rhamnose

Table 3. *Cell-wall composition in Corynebacterium pyogenes, C. haemolyticum and the large (L) colony mutants*

	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Glucosamine	Galactosamine	Unknown hexosamine	Alanine	Glutamic acid	Lysine
<i>C. pyogenes</i> , NCTC 5224	—	++	—	+	±	+	+	+	+++	+++	+++
<i>C. haemolyticum</i> , strain 53-W-1	—	++	—	—	—	+	+	+	+++	+++	+++
<i>C. pyogenes</i> , strain 637-s	—	++	—	+	tr	+	+	+	+++	+++	+++
<i>C. pyogenes</i> , strain 637-L	—	++	—	—	—	+	+	+	+++	+++	+++
<i>C. pyogenes</i> , strain 14-1-s	—	++	—	++	—	+	+	+	+++	+++	+++
<i>C. pyogenes</i> , strain 14-1-L	—	++	—	—	—	+	—	+	+++	+++	+++
<i>C. pyogenes</i> , strain 13081-s	—	++	—	++	—	+	+	+	+++	+++	+++
<i>C. pyogenes</i> , strain 13081-L	—	++	—	—	—	+	+	+	+++	+++	+++

No differences were found in the amino acid or hexosamine distributions in any of the strains except 14-1-L, in which galactosamine was not detected. The substance referred to as 'unknown hexosamine' in Table 3 (originally described by Strange & Powell, 1954) has recently been more fully characterized; its probable structure is 3-*o*- α -carboxyethyl-hexosamine (Strange, 1956; Strange & Dark, 1956).

*Precipitation tests with antisera prepared against
cell-wall fragments*

Crude cell-wall fractions prepared from *Corynebacterium pyogenes* NCTC 5224 were washed but not treated with trypsin or pepsin and were used as antigen for rabbit immunization. The antiserum obtained gave strong precipitin reactions with formamide extracts (Fuller, 1938) of whole organisms of the homologous strain. Similar extracts of strains 637-*s*, 13081-*s*, 14-1-*s* and six other strains of *C. pyogenes s* gave equally strong reactions with this serum. Extracts of strains 637-*L*, 13081-*L* and 14-1-*L* did not react, nor did an extract of 53-W-1, the type strain of *C. haemolyticum*.

Table 4. *Serological reactions of formamide extracts in relation to the sugars and hexosamines present in cell walls*

	Content of sugars and hexosamines							Reaction of formamide extracts. Sera			
	Rhamnose	Galactose	Glucose	Mannose	Glucosamine	Galactosamine	'Unknown' hexosamine	<i>C. pyogenes</i> , NCTC 5224	<i>C. pyogenes</i> , 637- <i>L</i>	<i>Streptococcus</i> sp., group A	<i>Streptococcus</i> sp., group G
<i>C. pyogenes</i> , NCTC 5224 (<i>s</i>)	++	-	+	±	+	+	+	++	-	-	++
<i>C. haemolyticum</i> , strain 53/W/1	++	-	-	-	+	+	+	-	++	-	-
<i>C. pyogenes</i> , strain 14/1/ <i>L</i>	++	-	-	-	+	-	+	-	++	-	-
<i>Streptococcus</i> sp., group A	++	-	-	-	++	-	+	-	-	++	-
<i>Streptococcus</i> sp., group G	++	++	-	-	+	+	+	+	-	-	++

Antiserum obtained following the immunization of rabbits with similar cell-wall fractions from strain 637-*L* did not give precipitin reactions with strains 637-*s*, 13081-*s*, 14-1-*s*, or any of the other *s* strains of *Corynebacterium pyogenes*. This 637-*L* antiserum gave clear-cut reactions with antigens prepared from strains 13081-*L*, 14-1-*L*, and *C. haemolyticum* 53-W-1. Extracts from both *s* and *L* strains uniformly failed to react with group A streptococcal antiserum, and this lack of reaction is of interest in the case of the *L* variants, in view of the rather close resemblance between their cell-wall compositions and that of *Streptococcus pyogenes* (see Table 4).

Dr R. E. O. Williams (Streptococcal Reference Laboratory, Colindale, London) kindly examined the reactions of these extracts with antisera to streptococci of other Lancefield groups, and found that extracts from *s* strains cross-reacted with several different group G sera, but not with other group sera. Extracts of *L* strains did not react with any grouping sera. The cross-reaction with group G sera was also given by extracts of a purified cell-wall

fraction from *Corynebacterium pyogenes* NCTC 5224. Since this fraction had been treated with trypsin and pepsin during its preparation, it seems unlikely that the reactions with group G antiserum were due to a minor antigen.

It has been shown conclusively by McCarty (1952) that the group antigen of *Streptococcus pyogenes* is the polysaccharide moiety of the cell walls, and this is presumably true also of streptococci of other groups, and of *Corynebacterium pyogenes* and *C. haemolyticum*. The sugar components found in the cell walls of two group G strains were rhamnose and galactose, together with three hexosamines (Cummins & Harris, 1956; and unpublished observations). The cross-reaction between these strains and *C. pyogenes*, and the lack of it in the case of strain 14-1-*L* and *S. pyogenes*, shows that there is little correspondence between qualitative chemical composition and immunological specificity. The latter property is presumably due more to the special arrangement of specific groups at the surface of the polysaccharide molecule than to the actual monosaccharide units themselves. This point is also illustrated in the work of McCarty (1956) on the serological specificity of cell-wall polysaccharides in a group A streptococcus and a variant of it whose polysaccharide did not react with group A serum. Both contained rhamnose and glucosamine as the principal components of the polysaccharide, but in the variant strain specificity seemed to depend on a rhamnose-rhamnose linkage, while in the parent this linkage was masked by side chains of *N*-acetylglucosamine which determined the original group A specificity.

DISCUSSION

It seems evident from the observations presented here that the bacterium described as *Corynebacterium haemolyticum* is actually a mutant form of *C. pyogenes*. The wild type, *C. pyogenes*, ferments lactose and xylose, produces a soluble haemolysin and/or proteinase and contains glucose in its cell wall, while the mutant *C. haemolyticum* ferments lactose, but not xylose, does not produce a soluble haemolysin, and glucose is not detectable in its cell-wall hydrolysates. Whether or not a single mutation could account for such a seemingly multiple change as that manifest in the *s* to *L* variation, is certainly open to question. There is a possibility that the colonial and haemolytic characters expressed in the *L* mutant may represent the culmination of a chain of mutational steps. On the other hand, perhaps the change in the cell-wall character $CW_{gluc^+} \rightarrow CW_{gluc^-}$ is the only change which takes place during the change from small to large colony form, and that the apparent alteration in fermentation pattern obtains because xylose is barred from reaching the interior of the cell as a result of the altered nature of the cell wall. On Mueller & Miller's semi-defined casein hydrolysate medium (1941) strain 637-*s* grows with xylose or glucose as a carbon source; strain 637-*L* does not grow with xylose as a carbon source but grows when glucose is present. It has been mentioned that one manner in which *s* and *L* colonies differ lies in the size of the haemolytic zone that develops around colonies on a blood plate. Both colonies are obviously haemolytic, but in the case of *L* the haemolysin never

reaches a detectable concentration in liquid medium. The ability to produce haemolysin may be the same in both the wild type and the mutant, but its liberation in detectable amounts may be possible only in the case of the wild type. The same phenomenon may account for the lack of proteolytic ability found in *L* cultures. We have already indicated the possibility that the haemolysin and the proteolytic enzyme are identical. That the change in cell-wall composition is a significant one is suggested by the lack of immunological cross-reaction between *s* and *L* strains.

There are other minor differences to be found between certain *s* and *L* pairs, some of which are recorded in Table 1. It is evident from Table 1 that all strains of *Corynebacterium pyogenes* and *C. haemolyticum* ferment lactose. We feel strongly that these organisms are 'lactic acid bacteria', probably streptococci; certainly they have little in common with other corynebacteria. The metabolism of *C. pyogenes* is that associated with facultative anaerobes and the pattern of its cell-wall components is quite different from that found in such corynebacteria as *C. diphtheriae* and *C. ovis*. The fact that *C. pyogenes* commonly assumes the morphology of a bacillus has no doubt been in large part responsible for its being placed initially among the corynebacteria. This morphological characteristic is an inconstant one, however, for under appropriate conditions these organisms grow as cocci in short chains (Brown & Orcutt, 1920).

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The Staining of Influenza Virus Filaments

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SUMMARY: Influenza virus filaments can be made visible in the ordinary microscope by various staining procedures; a technique involving potassium permanganate and Victoria blue is described.

Filamentous structures associated with influenza virus were first observed in electron micrographs by Mosley & Wyckoff (1946); Chu, Dawson & Elford (1949) later showed that the filaments could easily be seen in the dark-ground microscope and were particularly numerous in certain freshly isolated strains. The significance of these filaments is not yet fully understood. Some of the fundamental properties of the spherical particles of influenza virus are undoubtedly shared by the filaments: close association with virus infection, capacity to agglutinate red cells and to destroy cell receptors, possession of certain antigenic characters. Whether individual filaments are infectious is open to debate (Donald & Isaacs, 1954) and so is the question of their role in natural and experimental infection of man and animals.

Electron micrographs of influenza virus filaments have been published by many workers, dark-ground microphotographs by Hoyle (1954). The filaments have a very constant diameter, estimated from sections of bundles of adjacent filaments to be 50-60 m μ . (Morgan, Rose & Moore, 1956), and are variable in length reaching, in fresh allantoic fluid, up to 20 μ . and more (Burnet, 1956*b*). On dark-ground observation the filaments are remarkably stiff and show brilliant bodies of apparently larger diameter usually at one end, less commonly at both ends or along their length. These 'knobs' (Burnet, 1956*b*) can also be seen in the electron microscope; however, since electron microscopy is frequently carried out on virus eluates, which can hardly be prepared without breaking most of the filaments into shorter fragments (Bang & Isaacs, 1956), many of these pictures do not reveal the high degree of regularity with which terminal bodies can be found in fresh preparations. The filaments seem to be formed as protrusions from the surface of the allantoic cells (Bang, 1955); they are easily disintegrated by various chemical and physical agents (Donald & Isaacs, 1954; Burnet, 1956*a*).

The diameter of the filaments lies below the limit of resolution of the light microscope; in phase contrast, therefore, only aggregates of several agglutinated filaments can be seen. So far, nobody seems to have stained filaments in order to make them visible in an ordinary microscope. There is no obvious reason why this should not be possible, since bacterial flagella, which are much thinner, have been successfully stained for the past 70 years. This paper describes a staining method for influenza virus filaments from allantoic fluid.

Such a method can hardly be expected to throw an exciting new light on virus morphology; it might, nevertheless, prove useful if adaptable to normal histological technique.

METHODS

Virus. A/Persian Gulf/2/52, a strain of influenza virus which regularly produces a high proportion of filaments, was used in most experiments. MEL, an influenza A strain producing only very few filaments, and uninfected allantoic fluid served as controls. Ten-day-old eggs were infected by the allantoic route from stock capillaries containing seed kept at -70° and the fluids were harvested after 48 hr. of incubation at 35° . In the case of A/Persian Gulf/2/52 such fluids showed large numbers of filaments on dark-ground examination.

Virus eluates were prepared by absorbing 20 ml. allantoic fluid with 0.1 ml. of washed packed fowl red cells at 2° for 30 min., centrifuging the cells at 500 rev./min. for 5 min. and eluting into 10 ml. saline at 37° for 1 hr. Antisera were obtained from convalescent ferrets after intranasal infection.

Recommended procedure for staining

Preparation of slides. Scrupulously cleaned slides free from all traces of grease and dust are placed in a moist chamber and covered with 0.5 ml. freshly harvested allantoic fluid or virus eluate. After 1 hr., excess fluid is drained off by tilting the slide on to a piece of filter-paper and replaced by 0.5 ml. of buffered formalin (Herzberg, 1953). After 15 min. the formalin is poured off and the slide allowed to dry overnight at 37° .

Staining solutions. (A) 1% (w/v) aqueous potassium permanganate; (B) 20 parts of 3% (w/v) aqueous Victoria blue 4R + 1 part of saturated aqueous solution of citric acid. These reagents were prepared according to the recommendations of Herzberg (1953) and mixed immediately before use.

Staining schedule. Rapidly pass slide through a Bunsen flame; rinse in tap water; cover with 1 ml. solution (A) for 10 min., rinse; cover with 1 ml. solution (B) for 30 min., rinse; dry. Filaments appear dark blue-violet on light violet background; cell nuclei are blue-black.

Comment. The preparation must not be allowed to dry before the formalin fixation. Several batches of Victoria blue 4R from different manufacturers have been used with much the same results; however, with some batches, and particularly with freshly prepared solutions, the staining may require a much longer time. Sufficiently stained preparations are, when held against a white sheet of paper, of a perfectly even plain cobalt blue colour. Some immersion fluids bleach the stain in a matter of minutes and must be avoided; the same is true of many current mounting media. A convenient method is to mount the preparation in pure paraffin oil and to ring the coverslip with a paraffin-wax mixture. The proposed staining technique, derived from Herzberg's stain for Paschen bodies (Herzberg, 1953) and from Ruiter's stain for spirochaetes (Ruiter, 1938), lends itself to a number of variations. Thus, the buffered formalin may be replaced by osmium tetroxide vapour; the potassium per-

manganate may be omitted or replaced by 10% (w/v) aqueous mercuriochrome. This latter variation, yielding preparations in which the cell nuclei are bright red and the filaments pale violet, was suggested by the reports of Craigie (1933) and Vago (1947). Many stains recommended for flagella, Paschen bodies and Treponemata may be suitably adapted to influenza filaments; successful staining was obtained with Leifson's, Löffler's, Zettnow's and Morosow's methods. The silver impregnations, an example of which is given in Pl. 1, fig. 3, might prove useful in histology; they give, when successful, quite impressive pictures.

RESULTS

Plate 1, fig. 1, shows a preparation of stained influenza virus filaments from fresh allantoic fluid photographed in ordinary light. The terminal bodies or 'knobs', which are a striking feature in the dark-ground microscope, are easily recognized. Granules of similar appearance to the terminal bodies but not connected with the filaments, as well as smaller granules, are scattered throughout the field. Pl. 1, fig. 2, shows virus filaments adsorbed on to fowl red cell ghosts. This preparation, obtained by mixing red cell ghosts and infected allantoic fluid immediately before preparing the slide, gives a good idea of the length of the filaments and of the agglutination mechanism. Pl. 1, fig. 3, shows a virus eluate stained by Zettnow's silver impregnation (Craigie 1929). There is clearly no basic difference between the structures revealed by the Victoria blue and silver techniques. When large numbers of filaments from eluates and fresh allantoic fluids are compared, the former are found to be shorter, indicating that breakage occurs in the process of adsorption and elution. Pl. 1, fig. 4, shows for comparison an electron micrograph of the eluate used in fig. 3, treated with phosphotungstic acid (Hall, 1955) and adjusted to the same magnification. The large gain in resolution from electron microscopy can be more fully appreciated on the original films, from which a fourfold increase in apparent filament diameter with the preparation examined by light microscopy is evident.

No filaments similar to those described were found in uninfected allantoic fluids, in fluids infected with the MEL strain of virus, nor in eluates from this strain. The filaments in a preparation of A/Persian Gulf/2/52 virus could be removed by three cycles of absorption with fowl red cells. On addition of specific virus antiserum, agglutination of the filaments was observed. As a further control, the filaments were watched by dark-ground microscopy through the steps of fixation, mordanting and staining until they emerged into visibility by transmitted light.

DISCUSSION

The staining of viruses, mainly of the larger pox viruses, has been a controversial matter ever since the description of tiny granules in vaccinal exudates by Buist in 1887. That the elementary bodies demonstrable by the methods of Paschen, Morosow, Herzberg and others are actually virus corpuscles can no longer be doubted, though the general view about stained preparations is best

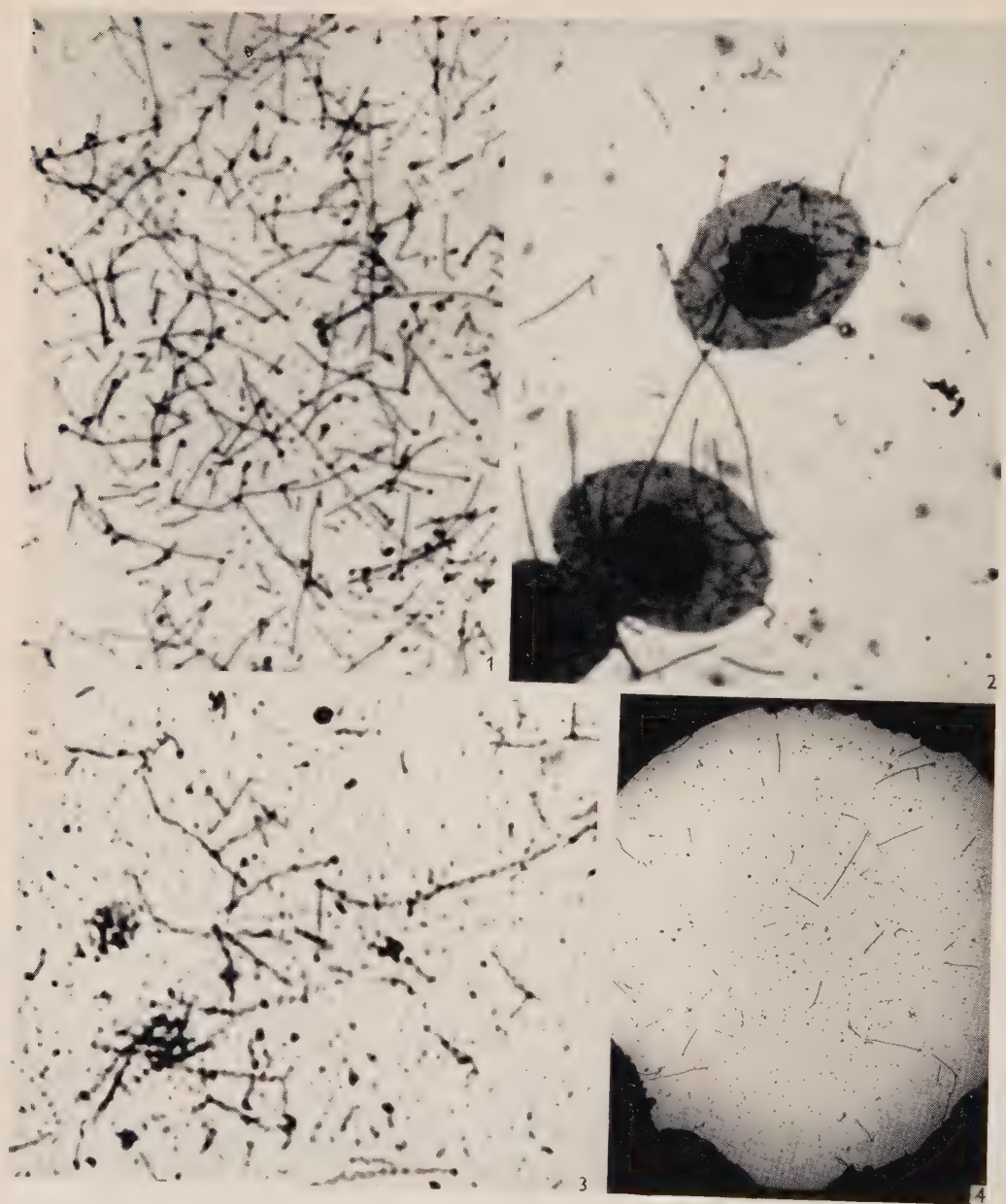
expressed in the words of Burnet & Andrewes (1933): 'All that can be said is that the normal particle size is less than the apparent size of the stained particles.' The difficulty with these very fine structures is not so much one of staining as one of discriminating between virus and background precipitates. This distinction is obviously much easier in the case of filamentous objects. The identity of the stained filaments with those seen in dark-ground and electron microscopy is supported by the following facts: they were found only in allantoic fluids infected with the filamentous strain; they could be removed from such fluids by red cells and recovered in the eluate; they showed agglutination after specific virus antiserum had been added. Finally, all the morphological characteristics of filaments known from electron and dark-ground microscopy are compatible with the stained picture; it is even surprising that such fine details as the terminal bodies, far from being blurred, are rather emphasized by staining. This is the more important since these bodies might represent, as suggested by Burnet (1956*b*) the actual infective 'warhead' of the filament.

It is much more difficult to identify the isolated granules seen in the same preparations. Their general aspect and distribution are strongly reminiscent of the spherical particles of influenza virus, but the unequivocal identification of a single granule is almost impossible, stained debris and precipitates being probably always present. From a histological point of view, however, the spheres are less interesting, since it is only from the filaments that we can ever hope to diagnose influenza virus in a tissue section or an impression smear.

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J. LINDENMANN—STAINING OF INFLUENZA VIRUS FILAMENTS. PLATE 1

(Facing p. 763)

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EXPLANATION OF PLATE

Figs. 1-3 were photographed with an apochromatic objective of 1.4 N.A., fig. 4 with a Siemens electron microscope. All figures $\times 2400$. Note increase in filament diameter produced by staining.

Fig. 1. Influenza virus from fresh allantoic fluid. Victoria blue.

Fig. 2. Adsorption of influenza virus on laked fowl red cells. Victoria blue.

Fig. 3. Influenza virus from an eluate. Zettnow's silver impregnation.

Fig. 4. Influenza virus from the same eluate as fig. 3. Electron micrograph of a preparation treated with phosphotungstic acid.

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Some Effects of Ultraviolet Radiation on the Pathogenicity of *Botrytis fabae*, *Uromyces fabae* and *Erysiphe graminis*

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SUMMARY: Ultraviolet irradiation of spores of three leaf-infecting fungi, *Botrytis fabae*, *Uromyces fabae* (causes of 'chocolate spot' and rust of broad beans, respectively) and *Erysiphe graminis* (cause of barley powdery mildew), decreased their pathogenicity, as assessed by counts of local lesions or pustules. The infectivity of *B. fabae* was lost more rapidly than the ability to form colonies on agar; with *E. graminis* infectivity was lost more rapidly than the ability to germinate. Ultraviolet radiation damage to spores of all three fungi was mitigated by exposure to daylight after irradiation. The extent of such photoreactivation of *B. fabae* was the same whether the spores were on the host plant or *in vitro*. Ultraviolet irradiation of leaves before inoculation decreased the number of pustules of *E. graminis* on barley, had no effect on the pustule number caused by *U. fabae* and increased the number of lesions caused by *B. fabae* on broad beans. Rubbing leaves with Celite before inoculation also increased the number of *B. fabae* lesions. Retaining u.v.-irradiated broad bean plants in daylight or darkness after inoculation with un-irradiated spores of *B. fabae* did not significantly alter the lesion number. In contrast, more pustules of *E. graminis* developed on u.v.-irradiated barley leaves kept in daylight than in darkness.

Ultraviolet (u.v.) radiation can affect micro-organisms in many ways, and some of the consequences can be mitigated by exposure to visible light afterwards. Kelner (1949) found that u.v.-irradiated bacteria and spores of fungi that would not grow when left in darkness, grew when illuminated. A comparable phenomenon, which has come to be called 'photoreactivation', has also been reported with bacteriophages (Dulbecco, 1950) and plant viruses (Bawden & Kleczkowski, 1955), but with these it occurs only when infected hosts are illuminated and not when the u.v.-irradiated viruses are illuminated *in vitro*. Exposure of leaves to u.v. radiation also affects their susceptibility to infection by plant viruses; for example, immediately after French bean leaves are irradiated, they resist infection with tobacco necrosis viruses, but when left in the light the leaves recover their initial susceptibility (Bawden & Kleczkowski, 1952) or may even become more susceptible than unirradiated leaves (Benda, 1955).

The loss of infectivity by spores of *Botrytis fabae* Sardiña caused by u.v. radiation can also be counteracted by exposing the spores to visible light (Last & Buxton, 1955). The work we describe now was done to compare the effects of u.v. radiation on the host and parasite relations of viruses with those for both obligately and facultatively parasitic fungi.

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METHODS

Broad bean with Botrytis fabae and Uromyces fabae (Pers.) de Bary Broad beans cv. Seville Longpod were grown in 8 in. pots containing John Innes potting compost and were used for experiments when 12–18 days old. Ultra-violet radiation, more than 95 % of which was wavelength 2537 Å., was provided by a Hanovia XII medium-pressure lamp (lamp A). Bean leaves were irradiated at 20 cm. from the tube of the lamp, and spores of the two fungi (0.2 ml. of dense suspensions in glass cavity slides of depth 1 mm.) at 40 cm. At these distances the radiation intensities were *c.* 692 and 173 $\mu\text{W./cm.}^2$ respectively. Spores of *Botrytis fabae* were used only from cultures 10–20 days old and the concentrations used in the water suspensions were increased for the older cultures to allow for their decreased infectivity (Last & Hamley, 1956). The spores were prepared from colonies grown at 20° on an agar medium constituted as follows (g./l. water): KH_2PO_4 , 5.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.2; KNO_3 , 2.0; CaCl_2 , 0.01; glucose, 20.0; peptone (Bacteriological, Hopkin & Williams Ltd.), 5.0; agar, (Hopkin & Williams Ltd.) 30.0.

Stock cultures of *Uromyces fabae* were retained by infecting beans with water suspensions of uredospores.

Inoculations to leaves were made by the method described by Last & Hamley (1956), and each bifoliate leaf was split for treatment into four half-leaflets, enabling a 4 × 4 Latin square experimental design to be done on plants with four leaves. When *Botrytis fabae* was used, the leaves, attached to part of the stem, were detached from the plant, stood in water and placed in a saturated atmosphere under a glass bell-jar. Lesions were counted after incubation for 48 hr. at *c.* 20° and their numbers (*x* lesions/half-leaflet) were transformed to *z*, where

$$z = \log_{10} \frac{1}{2}[x + c + \sqrt{(x^2 + 2cx)}] \quad \text{and} \quad c = 20 \quad (\text{Kleczkowski, 1955}).$$

Leaves inoculated with *Uromyces fabae* were not detached; plants were kept in a saturated atmosphere under bell-jars for 4 days, then moved into the glasshouse. Colony counts were made after 10 days.

Barley with Erysiphe graminis DC. For germination and infectivity tests with *E. graminis* DC, either dry microscope slides or the upper surfaces of detached leaves were coated with conidia shaken from infected leaves held above them, and were then incubated at room temperature, *c.* 15°. The detached barley leaves cv. Plumage Archer, each attached to 1.5 cm. of its sheath, were stood singly in test tubes filled with tap water to a depth of 1 cm.; the slides were put in a saturated atmosphere in Petri dishes.

For u.v.-irradiating *Erysiphe graminis*, a different lamp was used; it was a Hanovia medium-pressure lamp giving u.v. radiation of which more than 95 % was of wavelength 2537 Å. and an intensity of 108 $\mu\text{W./cm.}^2$ when 20 cm. above the leaves and slides (lamp B). Only half of the leaf or coated slide was irradiated and the unexposed half acted as a control. When testing for inactivation by u.v. radiation, the leaves inoculated with spores were kept in

darkness for 2 days and then exposed to daylight until pustules were counted; the slides were kept in darkness until germination counts were made. The zones within 1 cm. of the boundary were ignored.

The percentage germination after 2 days on the u.v.-irradiated section of the slide was corrected to allow for the incomplete germination of the un-irradiated spores. The effect on infectivity was measured after 5 days by expressing the number of colonies/unit area of exposed section of leaf as a percentage of the number on its complementary unexposed section. All treatments were replicated at least five times.

RESULTS

Survival curves after ultraviolet irradiation

Agar plates, each spread with 1000 spores of *Botrytis fabae*, and cavity slides containing spore suspensions of either *B. fabae* or *Uromyces fabae* were irradiated for different periods of time. Subsequently, the agar plates were incubated in darkness at 22.5°, and the suspensions within the cavity slides were inoculated to broad bean leaves which were then also kept in darkness. Because the numbers of lesions on bean leaves are not simply related to the inoculum concentration, a dilution series of unirradiated spores of *B. fabae* was made at the same time. From it the percentage survivals, which were plotted on a logarithmic scale (Fig. 1A), were derived.

The decrease in viability (ability to form colonies on agar) and of infectivity (ability to form lesions on the host plant) did not follow the course of a first-order reaction, i.e. the logarithms of proportions of spores viable and infective plotted against the times of irradiation did not give straight lines. The lines were at first almost horizontal and then turned downwards to approach asymptotically straight lines, resembling the course of so-called 'multiple hit' curves. Logarithmic estimates of 'multiplicity', obtained by extrapolating the descending straight parts of the curves to the ordinate, show that these spores behave as if c. 8 centres had to be affected to make them non-viable, and only three to four centres to make them non-infective.

An exposure of 1 min. to u.v. radiation made a greater proportion of *Botrytis fabae* spores non-infective than of *Uromyces fabae* spores (Fig. 1B). These data are the actual numbers of lesions or pustules/half-leaflet, and have not been corrected for percentage survival. In contrast to *B. fabae* the decline in germination and infectivity of conidia of *Erysiphe graminis* with increasing exposure to u.v. radiation seems to follow the course of a first-order reaction at least with exposures up to 1 min. Like *B. fabae*, however, the viability of *E. graminis* *in vitro* was less affected by u.v. radiation than was its infectivity. Although the effects of u.v. radiation on the infectivity of *E. graminis* were measured on conidia already inoculated to barley leaves, results described later show that the decline in pustule number caused by exposure of up to 1 min. cannot be attributed to changes in the host's susceptibility.

Although the conidia of *Botrytis fabae* were in suspension, and those of *Erysiphe graminis* were not, the exposures necessary to decrease germination

by 50% probably reflect the different sensitivities of the two fungi, 20 and 127 sec. for *E. graminis* and *B. fabae*, respectively (Fig. 1C).

Survival curves, on agar only, were also obtained for *Aspergillus niger*, *Fusarium oxysporum* f. *pisi* races 1 and 2 and *Fusarium solani* f. *pisi*. Of these, the survival curves of *F. oxysporum* f. *pisi* races 1 and 2, which consisted

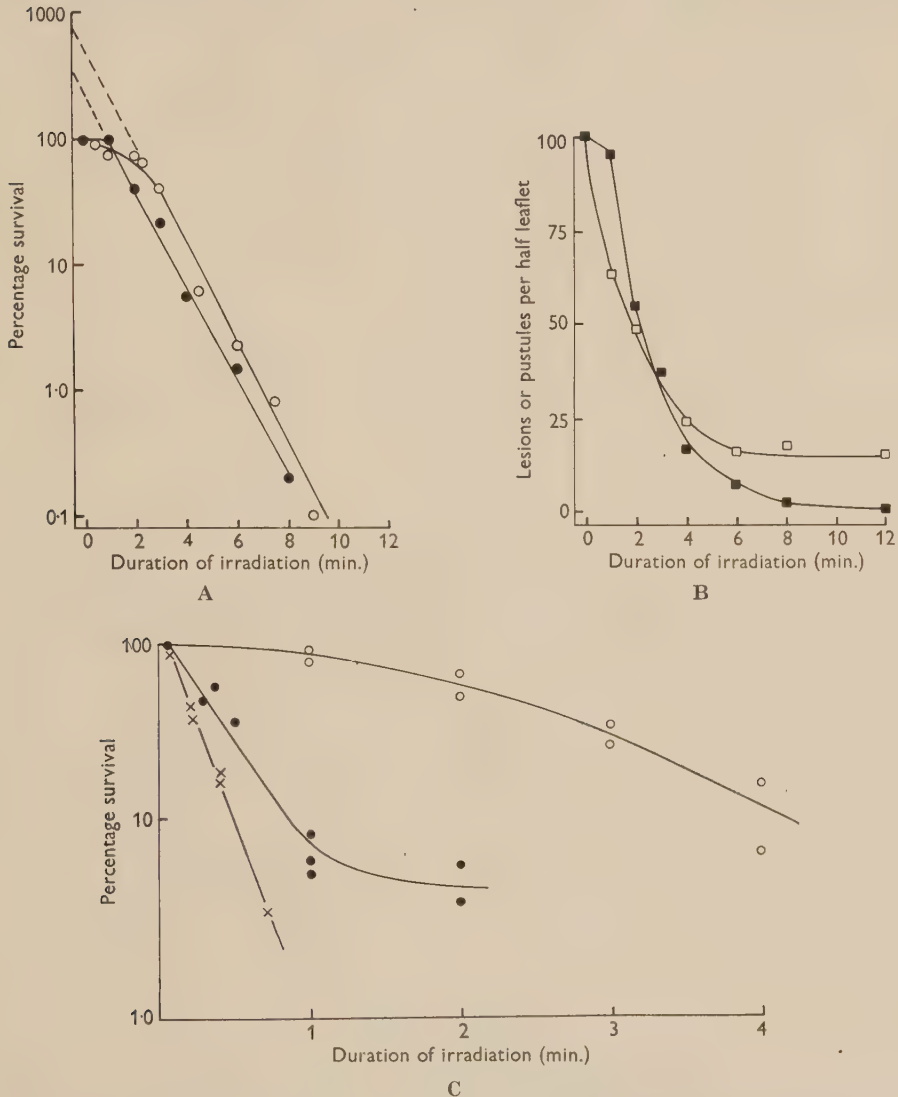


Fig. 1. Ultraviolet radiation survival curves of *Botrytis fabae* and *Uromyces fabae*. (A) Relation between time of exposure to lamp A and % survival of *B. fabae*. ● = survivors forming lesions on bean leaves; ○ = survivors forming colonies on agar media. (B) Relation between time of exposure to lamp A and number of lesions or pustules formed/half-leaflet by *B. fabae* and *U. fabae*. ■ = *B. fabae*; □ = *U. fabae*. (C) The effect of ultraviolet radiation with lamp B on the germination of conidia of *Erysiphe graminis* (●) and *B. fabae* (○), and on the infectivity of *E. graminis* (×).

mainly of uninucleate microconidia, followed the course of a first-order reaction, whereas those of *A. niger* and *F. solani* were similar to those obtained for *Botrytis fabae*.

When *Botrytis fabae* spores were u.v.-irradiated at different times after inoculation to leaves, the survival curves varied with the delay (Fig. 2). After each period of post-inoculation delay, leaves were u.v.-irradiated for 0, 2, 4 and 8 min. When the irradiation was immediately after inoculation, the

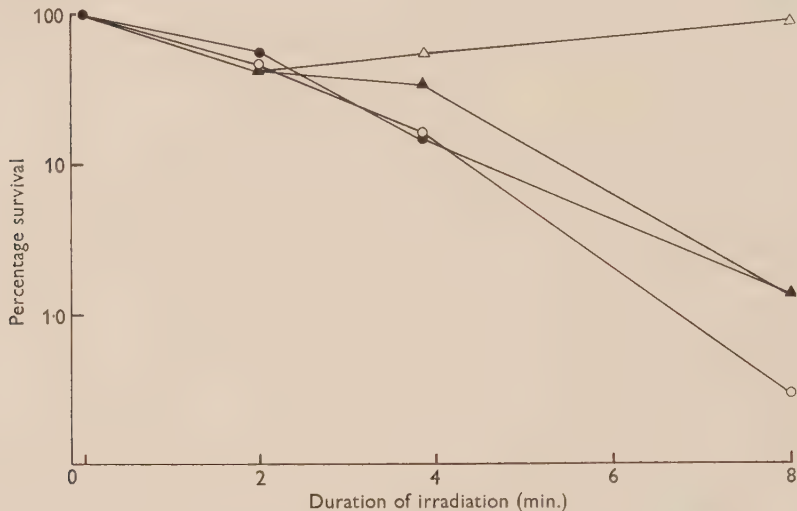


Fig. 2. Effect of u.v.-irradiating broad bean leaves for various times, at different intervals after inoculation with *Botrytis fabae*, on the numbers of lesions formed. Time after inoculation when irradiated o—o, 0 hr.; ●—●, 2 hr.; ▲—▲, 4 hr.; △—△, 7 hr.

number of lesions, expressed as a percentage of those formed without any irradiation, decreased as the time of irradiation increased. Irradiation 7 hr. after inoculation had no effect, presumably because spores had by then germinated and the fungus had penetrated the host, where it was shielded from the u.v. radiation. With *Erysiphe graminis*, exposure to u.v. radiation was less damaging when done 70 hr. after inoculation than after 20 hr. (Table 3). Comparable effects were obtained by Bawden & Harrison (1955) when French bean leaves were u.v.-irradiated at different intervals after they had been inoculated with a tobacco necrosis virus.

Photoreactivation

Exposure of spores of *Botrytis fabae* u.v.-irradiated for 3.5 min. (10% survival in Fig. 1A) to daylight before inoculation to plants, which were then kept in darkness, increases the infectivity of the spores as measured by lesion counts (Last & Buxton, 1955). Table 1 shows that photoreactivation occurred to the same extent whether the spores were exposed to daylight on inoculated leaves (A) or in cavity slides before they were inoculated to leaves (B).

The effects of u.v. radiation on spores of *Uromyces fabae* (Table 2) and *Erysiphe graminis* (Table 3) were also mitigated by exposure to daylight. When

irradiated suspensions, kept in cavity slides in darkness or light for 7 hr. were inoculated to plants subsequently kept in darkness for 4 days, 5 and 17 pustules of *U. fabae*/half-leaflet developed in one experiment and 5 and 10 in another. When the inoculated plants were kept in daylight, u.v.-irradiated spores kept in the light for 7 hr. before they were used as inoculum produced no more infections than spores kept in darkness. This implies that photo-reactivation occurred on the plants and that 7 hr. delay before exposure to light did not diminish the extent of photoreactivation.

Table 1. *The effect of exposing u.v.-irradiated spores of Botrytis fabae to daylight on the host plant and in vitro*

	No. of lesions/half-leaflet. Treatment of spores for 7 hr. after irradiation		
	Light, <i>L</i>	Dark, <i>D</i>	Effect of light (<i>L</i> - <i>D</i>)
A. Suspension inoculated to beans immediately after irradiation	(2.16) 125	(1.75) 38	(0.41)
B. Suspension left in slides for 7 hr. after irradiation before being inoculated to leaves	(2.37) 214	(2.01) 83	(0.36)

Figures in parentheses: transformed data. Significant difference between means of transformed data for $P=0.05$ is 0.17.

Table 2. *Effects of exposing u.v.-irradiated Uromyces fabae spores to daylight for 7 hr. before inoculation to beans, and of subsequently keeping the inoculated plants in light or darkness*

	No. of pustules/half-leaflet	
	Exp. 1	Exp. 2
Pre-inoculation treatment	Plants in daylight after inoculation	
Spores in daylight	24	13
Spores in darkness	20	13
	Plants in darkness after inoculation	
Spores in daylight	17	10
Spores in darkness	5	5

Last & Buxton (1955) found that exposure of *Botrytis fabae* spores for 2 hr. in cavity slides to daylight immediately after u.v. irradiation at least doubled the number of lesions formed when they were inoculated to bean leaves. When, however, irradiated spores were photoreactivated immediately after inoculation to leaves, 5.5 hr. was needed to produce a significant increase in lesion number, and a further 5 hr. produced no further increase.

The relative effect of photoreactivation increased as the exposure of *Botrytis fabae* and *Erysiphe graminis* spores to u.v. radiation increased. After 3 min. u.v. irradiation the ratio of the number of lesions formed by *B. fabae* spores in cavity slides in light to those kept in darkness for 7 hr. before inoculation to plants (which were then placed in darkness) was 2.6; after 7 min.

Table 3. *The effect of exposing u.v.-irradiated conidia of Erysiphe graminis to daylight on (a) germination and (b) infectivity*

(a) Effect on germination

Post-irradiation treatment	Period of exposure to u.v. radiation		
	5 sec.	20 sec.	60 sec.
	Germination (%)		
Light	100	98	100
Dark	98	60.2	8.3

(b) Effect on infectivity

Time after inoculation when irradiated (hr.)	Post-irradiation treatment	Duration of exposure to u.v. radiation		
		10 sec.	20 sec.	60 sec.
		No. pustules from irradiated conidia as % from unirradiated (transformed to angles for analysis)		
20	Light	73.1	82.2	78.6
	Dark	29.9	21.8	0.0
70	Light	65.0	74.1	75.4
	Dark	69.7	48.8	26.3

Significant difference between means of transformed data for $P=0.05$ is 21.6 (original data)

20	Light	85.9	95.3*	90.8*
	Dark	30.2	17.2	0.0
70	Light	81.8	83.6	89.1
	Dark	81.2	56.4	24.5

* Figures referred to in text.

Table 4. *The effect of light and dark post-irradiation treatments on the numbers of lesions formed/half-leaflet by spore suspensions of Botrytis fabae exposed to u.v. radiation*

Post-irradiation treatment	Duration of exposure to u.v. radiation (min.)								Mean
	3	4	4.5	5	5.5	6	6.5	7	
	Lesions/half-leaflet (transformed data)								
Light	1.74	1.76	1.88	1.74	1.63	1.67	1.51	1.55	1.69*
Dark	1.49	1.32	1.24	1.20	1.17	1.11	1.12	1.12	1.22
Mean†	1.61	1.54	1.56	1.47	1.40	1.39	1.32	1.34	—

* Significant difference between means for $P=0.05$ is 0.05.

† Significant difference between means for $P=0.05$ is 0.09.

Detransformed means									
Light	37	39	57	37	25	29	15.5	18	—
Dark	14	5.5	3	2	2	1	1	1	—
Ratio of light/dark									
	2.6	7.1	19.0	18.5	12.5	29.0	15.5	18.0	—

u.v. irradiation the ratio was 18 (Table 4). With *E. graminis*, photoreactivation of spores u.v.-irradiated for 20 sec. increased pustule number from 17 to 95% of pustules formed by unirradiated spores and, after u.v. irradiation for 60 sec., photoreactivation increased pustules from 0 to 90% (Table 3).

The relatively greater effects of photoreactivation with increasing degree of u.v. irradiation has been found with all biological systems tested and has been called 'the dose reduction principle' (Kelner, 1949). The effects of u.v. irradiation on the fungi used in our experiments differ from the results obtained in similar work on bacterial and plant viruses. Viruses can be photoreactivated only after they have infected host cells (Dulbecco, 1950; Bawden & Kleczkowski, 1953), whereas fungi, whether obligate or facultative parasites, can be photoreactivated *in vitro*. The capacity to do this may lie in cell components of fungus spores which are lacking in the non-cellular viruses.

Effects of ultraviolet radiation on leaf susceptibility

Bawden & Kleczkowski (1952) showed that u.v. irradiation of French bean leaves before inoculation increased their resistance to infection by tobacco necrosis virus, and that susceptibility was regained by their subsequent exposure to daylight. Similarly, u.v. irradiation increased the resistance of barley leaves to infection with *Erysiphe graminis*, and again, this resistance was lowered by their subsequent exposure to daylight. A minimum of 4 min. u.v. irradiation was necessary to detect this effect (Table 5). In contrast, u.v. irradiation of broad bean leaves for 4 min. before inoculation increased both the number and the size of lesions formed by *Botrytis fabae* (Table 6); it did not affect the lesions produced by *Uromyces fabae*.

Table 5. *Effects of irradiating barley leaves before inoculation with Erysiphe graminis, and of subsequent exposures to daylight and darkness on numbers of pustules developing*

Post-irradiation treatment	Duration of exposure to u.v. radiation (min.)				
	1	2	4	6	8
	Numbers of pustules/unit area on irradiated section as % of pustule number on unirradiated section				
Daylight	100	100	42.7	11.5	0.0
Darkness	100	89	0.0	2.2	0.0

Table 6. *Effects on bean leaves of (a) rubbing with Celite and (b) u.v. radiation before inoculation with Botrytis fabae*

(a) Number of lesions/half-leaflet				
	With Celite		Without Celite	
	184		82	
(b) Ratio of lesions on irradiated to unirradiated half-leaflets				
	Duration of exposure to u.v. radiation (min.)			
	1	2	4	8
Ratio	1.2	2.2	2.6	3.4

Increasing the u.v. irradiation of the leaves from 1 to 8 min. progressively increased the susceptibility of bean leaves to *Botrytis fabae*. With *Uromyces fabae*, which needed 10 days to cause lesions to appear, compared with 1 day with *B. fabae*, similar observations were not possible because leaves irradiated for 4 min. bronzed and died. Placing u.v.-irradiated plants in light or darkness after inoculation with *B. fabae* did not significantly alter their susceptibility.

The effect of irradiating leaves before inoculation on the number of lesions given by *Botrytis fabae* was similar to that caused by rubbing the leaves with a Celite paste immediately before inoculation (Table 6). Both u.v. radiation and rubbing with Celite may act by altering the leaf cells so that substances which promote infection by *B. fabae* are exuded in greater amount. Stimulation of infection by foliar exudates was demonstrated by Brown (1922) with *B. cinerea*, and Norell (1954) showed that irradiation of potato disks with u.v. radiation increased the exudation of substances which stimulated the growth of *Fusarium culmorum*. In attempting to explain this, Norell considered that a vital substance in the cell became oxidized by oxidizing agents formed as a result of the u.v. irradiation, or possibly by a direct photo-oxidative process. If such a substance, in its reduced state, maintained cell permeability, the u.v. irradiation might cause increased permeability, thus leading to increased exudation from the cells. If this occurred with irradiated bean leaves, once sufficient cell exudate was present on the leaf surface to stimulate infection by *B. fabae* spores, it seems unlikely that subsequent exposure of the leaves to daylight would have any effect on their susceptibility. The obligate parasites *Uromyces fabae* and *Erysiphe graminis* are less likely than *B. fabae* to respond to leaf exudates and infection by these organisms probably depends on the internal metabolism of the leaf cells. Like plant viruses, successful infection by them may depend on the nucleoprotein metabolism of the host cell, and if this were impaired by u.v. irradiation, the leaves would become resistant.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its twenty-third General Meeting in the University of Exeter on Thursday and Friday, 20 and 21 September 1956. The following communications were made:

COMMUNICATIONS

Changes Occurring in the Composition of *Aspergillus nidulans* Conidia during Germination. By C. J. SHEPHERD (*M.R.C. Unit for Chemical Microbiology, University of Cambridge*)

In resting conidia, 63 % of the total phosphorus is in the form of polymetaphosphate, 33 % is organically bound and 4 % is present as orthophosphate. After 8 hr. incubation at 37° in a medium allowing germination, the polymetaphosphate fraction accounts for only 2 % of the total phosphate, the orthophosphate rising to 92 % of the total phosphate present in the mycelium; the remaining 6 % is accounted for as organically bound phosphate.

Considerable changes occur in the distribution of cell nitrogen during the transition of resting conidium to mycelium. The acid-soluble nitrogen, expressed as a percentage of the total nitrogen present, rises from 7 % in the resting spore to 22 % in the mycelium. Similarly, the protein nitrogen rises from 26 % of the total in the spore to 36 % in the mycelium. There is a concomitant fall of cell-wall nitrogen from 21 to 10 % and of RNA-nitrogen from 46 to 33 % of the total present, while the proportions of DNA-nitrogen and lipid nitrogen are virtually unchanged.

Resting conidia contain a free amino acid pool, the major components of which are glutamic acid, aspartic acid, glutamine and γ -aminobutyric acid. After 1 hr. incubation at 37° in a medium allowing germination there is a rise in the amounts of glutamic and aspartic acids present and a complete disappearance of the asparagine, while the concentration of the other amino acids falls. After 3 hr. γ -aminobutyric acid has also disappeared, and the initial fall in the concentration of the other amino acids is followed by increased synthesis. After 6 hr. the rates of synthesis of the various amino acids are approximately equal.

During the pre-vegetative phase of germination there is a slow synthesis of RNA and protein, with a concomitant utilization of acid-soluble nitrogen and a breakdown of cell-wall material. The commencement of the vegetative phase is marked by a rapid synthesis of RNA and protein, while the synthesis of DNA and cell-wall material commences after 4 hr. After 5 hr. the rates of synthesis of protein, DNA, RNA, cell-wall and acid-soluble nitrogen are approximately equal. During the first 9 hr. of germination there is a progressive fall in the total lipid content of the spore.

The Utilization of Raffinose by *Lactobacillus bifidus*. By R. P. LLOYD
(*Department of Biochemistry, University of Cambridge*)

An anaerobic strain of *Lactobacillus bifidus*, isolated from babies' faeces, grew in peptone-water indicator broth containing glucose, galactose, maltose, lactose or raffinose, forming acid but no gas. There was no growth or acid production with fructose or sucrose or in the absence of sugar. Washed suspensions of the bacteria in bicarbonate buffer in Warburg manometers formed acid from glucose, galactose, lactose and raffinose, and also from melibiose and stachyose. Acid production in the presence of sucrose or fructose was no more than that in the absence of sugar. Complete utilization of the raffinose molecule was indicated by the fact that the yield of acid from this sugar was three times as great as that from equimolar amounts of glucose. Acid production ceased in all cases when the bacteria were treated with cetyltrimethylammonium bromide at 20 $\mu\text{g./mg.}$ dry weight, but paper chromatography revealed that raffinose was metabolized with the formation of free galactose, sucrose and fructose, and of glucose phosphates. Such detergent-treated cells also degraded added sucrose to glucose phosphates and fructose in the presence of phosphate, or into free glucose and fructose in the presence of arsenate, indicating the operation of sucrose phosphorylase (Doudoroff, Barker & Hassid, 1947).

These results suggest that sucrose and fructose, although not utilized by intact cells, are intermediates in the metabolism of raffinose.

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Genetic Recombination in *Streptomyces coelicolor*. By D. A. HOPWOOD
(*Botany School, University of Cambridge*)

A small percentage of spores from a mixed growth of two multiple auxotrophic mutant strains produced stable prototrophic colonies; spores from the parents grown separately produced none. Recombination rather than mere diploidy or heterokaryosis was shown by isolating, on suitably supplemented media, strains having some of the nutritional deficiencies of both parents. Thus the results of Sermonti & Spada-Sermonti (1955) have been independently confirmed using a different strain of *Streptomyces coelicolor*.

Crosses involving four selective markers have yielded quantitative recombination data. By using a series of selective media, at least one member of each of the seven pairs of complementary recombinant genotypes expected amongst the progeny can be isolated and their relative frequencies determined. These frequencies can be used to construct a linkage map of the four markers, assuming as a working hypothesis that recombination involves the fusion of two complete nuclei followed by a reduction process in which complementary genotypes arise in equal numbers. The map can be expressed in terms, not of the percentage of recombination between markers, but only of the relative

frequency of recombination in the different regions. The results of several crosses have given an unequivocal order of the markers used. In those cases where both members of a complementary pair of genotypes can be recovered, the assumption that they arise in equal numbers has been confirmed.

Thus the recombination process must involve the transfer at least of large groups of linked characters, if not of complete nuclei.

It seems likely that nuclear fusion and reduction occur somewhere between hyphal fusion in the substrate mycelium, allowing heterokaryosis (Bradley & Lederberg, 1956), and spore production in the aerial hyphae.

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A New Infective Factor Controlling Sex Compatibility and Gene Recombination in *Escherichia coli*. By HELEN L. BERNSTEIN (*Lister Institute of Preventive Medicine, London*)

Escherichia coli strain WG3 has an infective factor controlling sex compatibility which resembles the infective agent F in *E. coli* strain K12. The two agents (F3 from WG3, and F12 from K12) were compared against a common genetic background to study the degree of fertility each conferred, the differences in gene recombination dependent on parental mating type, and the inheritance of each agent among recombinants.

Two K12 F⁻ auxotrophs, W1956 (T-L-B₁-Lac-Gal-Mal-Xyl-Mtl-Ara-S^RV₁^RV₆^R) and CL61 (M⁻) were separately infected to give an F3⁺ and an F12⁺ in each line in addition to the F⁻. Then all possible crosses were made, on Davis minimal medium.

With 2×10^8 each parent per plate, prototroph yield depended on mating types present. F12⁺ \times F⁻ and F12⁺ \times F12⁺ gave about 2 prototrophs per 10^6 M-cells plated, while F3⁺ \times F⁻ were only half as productive, and F3⁺ \times F3⁺ were the least fertile ($6/10^7$). F3⁺ \times F12⁺ gave the highest yields ($4-8/10^6$).

Analysis of 200 prototrophs from each cross gives the same gene order for seven unselected markers tested: (M) Mtl Xyl Mal S Gal Ara Lac (TL), but the frequency of recombination in any region depends on the F used and on the parent bearing it. Most progeny of F12⁺ \times F⁻ resemble the F⁻ parent. But most progeny of F3⁺ \times F⁻ resemble the M⁻ parent regardless of which parent is F⁺ or F⁻. And in all F3⁺ \times F3⁺, F12⁺ \times F12⁺, and F3⁺ \times F12⁺ crosses, the M⁻ parent makes the greater contribution. Thus, the 'donor-receptor' concept of mating type function in these bacteria here applies only to the F12⁺ \times F⁻ system.

The agents also differed in their inheritance; from F3⁺ \times F⁻, progeny are 10-30% F⁻. Currently being investigated is the problem of whether these differences between the two agents are expressed only at the genetic level, or also at physiological levels, perhaps in the mating process itself.

A Qualitatively Altered Enzyme in a *Neurospora crassa* Mutant. By
J. R. S. FINCHAM (*Department of Genetics, University College of Leicester*)

Several strains of *Neurospora crassa*, all the result of mutation at the *am* locus, appear to lack glutamic dehydrogenase and requires exogenous α -amino nitrogen for normal growth. Pateman has obtained numerous apparent back-mutations in *am* strains by treatment with ultraviolet light. Most of the back-mutant strains were indistinguishable from the wild type, but several showed exceptionally low glutamic dehydrogenase activities. One of these latter strains has been shown to correspond genetically to a new allele at the *am* locus (*am*^l), and it produces a glutamic dehydrogenase distinguished from the normal enzyme in being practically inactive when kept at 20° but capable of being activated by warming to 35°–50° for a few minutes. On returning the preparation to 20° the extra activity disappears in 1–2 hr. but can be regained by repeated heat treatment. Some activity of the enzyme can also be achieved by incubation with α -ketoglutarate and TPNH at 20°. The activity of glutamic dehydrogenase from the wild type is quite unaffected by these treatments. Experiments on the behaviour of mixed wild type and mutant preparations, and on the kinetics of the activation and decay reactions, indicate a reversible change in the mutant enzyme itself rather than a reaction with an impurity in the preparation. The growth of *am*^l-mutant strains shows a correlation with the properties of the enzyme which they contain; whereas at 25° they grow almost like the wild type, at 20° they require α -amino nitrogen for normal growth.

Bacterial Lysozyme. By M. H. RICHMOND (*Department of Biochemistry, University of Cambridge*)

An organism that will lyse a cell-wall preparation of *Micrococcus lysodeikticus* has been isolated from soil using the techniques devised by Salton (1955). The organism has been identified as a member of the genus *Bacillus*, probably a strain of *B. subtilis*. It grows well in liquid culture in a simple synthetic medium and under these conditions a lytic factor is released into the supernatant in the course of exponential growth. A comparison has been made between the properties of egg-white lysozyme and the lytic enzyme produced by the organism.

There is good agreement between the lytic actions of the two enzymes on intact cell, heat-killed cell and cell-wall preparations of *Micrococcus lysodeikticus*, *Sarcina lutea*, *Bacillus megaterium*, *B. cereus* and *B. subtilis*. The turbidity of a suspension of heat-killed cells of the organism producing the lytic factor is reduced by about 17% by incubation with a cell-free preparation of the same factor or egg-white lysozyme. Intact cells are not significantly affected.

Salton (personal communication) has found that cell walls of *Micrococcus lysodeikticus* are rendered completely soluble by egg-white lysozyme and that the soluble material is about 50% dialysable on a dry-weight basis. If similar cell-wall preparations are lysed to completion with the lytic factor,

the soluble products are 47% dialysable. Boundary electrophoresis of the non-dialysable fraction in veronal buffer at pH 8.0 shows a similar pattern whether lysis is achieved using egg-white lysozyme or the lytic factor under otherwise identical conditions.

On the basis of these biological properties the lytic enzyme produced by this strain of *Bacillus subtilis* can be characterized as a lysozyme.

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The Effect of Actidione on Protein and Nucleic Acid Synthesis in *Saccharomyces mandshuricus*. By D. KERRIDGE (*Department of Biochemistry, University of Cambridge*)

The antifungal antibiotic actidione was isolated by Whiffen, Bohonas & Emerson (1946) from *Streptomyces griseus* and the structure determined by Kornfeld & Jones (1948).

The test organism used throughout this study has been *Saccharomyces mandshuricus*, the growth of which is inhibited by actidione at concentrations of between 0.5 and 1.0 $\mu\text{g./ml.}$ At growth inhibitory concentrations there is no effect on respiration or fermentation, and it is possible to show an increase in the soluble 7' acid labile phosphate during inhibition of growth by actidione. It is therefore unlikely that the growth inhibitory activity of actidione is due to an interference with the energy producing mechanisms of *S. mandshuricus*.

Investigations of the effect of actidione on the synthesis of nucleic acid and protein by both logarithmically growing cells and washed suspensions have shown that at the growth inhibitory concentration, protein synthesis is completely inhibited, whereas the synthesis of nucleic acid, although reduced, can continue for a short period of time after the addition of the antibiotic. On fractionating the nucleic acid by the method of Schmidt & Thannhauser (1945), it was found that the residual synthesis of nucleic acid was due to the synthesis of ribonucleic acid only and that deoxyribonucleic acid synthesis was completely inhibited.

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SCHMIDT, G. & THANNHAUSER, S. J. (1945). *J. biol. Chem.* **161**, 83.
WHIFFEN, A. J., BOHONAS, N. & EMERSON, R. L. (1946). *J. Bact.* **52**, 610.

Protein Synthesis in a Thermophilic Bacillus. By R. HANCOCK (*Department of Biochemistry, University of Cambridge*)

The optimum conditions for protein synthesis, in particular the effect of temperature, have been investigated in a species of thermophilic bacillus; the incorporation of ^{14}C glycine into cell protein and the formation of certain enzymes have been used as measures of protein synthesis.

In washed cell suspensions linear incorporation of ^{14}C glycine into the protein fraction requires a complete amino acid mixture and an energy source, and is stimulated three- to four-fold by addition of a purine-pyrimidine mixture. Incorporated radioactivity is found solely in the glycine residues of the protein; it has not been possible to displace incorporated ^{14}C glycine by ^{12}C glycine. The optimum temperature for incorporation is the same as that for cell growth, approximately 55° , and is higher than that found for similar systems in mesophilic organisms.

Optimum conditions for the formation, in cell suspensions, of catalase and of the enzyme complex 'maltozymase' are the same as those for the incorporation of glycine; again the presence of a purine-pyrimidine mixture is markedly stimulatory. The rate of synthesis of catalase is maximal at 45° , and that of 'maltozymase' at 55° ; in both cases the optimum temperature is considerably higher than that for synthesis of enzymes in mesophilic cells.

The loss of activity of β -galactosidase and catalase from cells suspended in buffer at 55° can be prevented by addition of amino acids, purines, pyrimidines and glucose to the system; the further addition of chloramphenicol ($50\text{ }\mu\text{g./ml.}$) abolishes this stabilizing action. Under conditions of inactivation no breakdown of protein occurs as determined by release of radioactivity from ^{14}C labelled cells. These results support the theory put forward by Allen (1953) suggesting an equilibrium between enzyme inactivation and synthesis in thermophilic organisms.

REFERENCE

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Thermal Inactivation of a 'Marine' Bacteriophage. By R. SPENCER (Humber Laboratory, Food Investigation Organization, Department of Scientific and Industrial Research, Hull)

A bacteriophage, isolated from marine sources and active against a group of marine bacteria (Spencer, 1955), was completely inactivated within 10 min. when suspended in sea-water peptone water and exposed to a temperature of 50° . There was slight inactivation at 40° .

This degree of thermal sensitivity was greater than any reported for *terrestrial* bacteriophages, thus supporting the thesis that there is a difference between *marine* micro-organisms and *terrestrial* micro-organisms specifically related to their habitat.

Further experiments on the thermal inactivation at 40° showed it to depend markedly upon the nature of the suspending medium. Minimal inactivation took place in sea water or sea-water based nutrient media. The rate of inactivation was slightly greater in tap-water based nutrient media and considerably greater in distilled water. Inactivation in sodium chloride solutions to a concentration of 2.75 % was not markedly different from that in distilled water. Inactivation in magnesium chloride solutions was considerably less than in sodium chloride solutions but greater than in sea water. Inactivation in a solution of 2.75 % sodium chloride and 0.495 % magnesium chloride, the

approximate concentrations of these two cations, as their chlorides, in sea water, was, however, very similar to the inactivation in sea water itself. It was concluded that the protective effect of sea water in thermal inactivation was not due to an inherent virtue of sea water or to its sodium chloride content, but to a 'balanced salts action' shown to take place with terrestrial bacteriophages (Burnet & McKie, 1930).

The order of the thermal inactivation of the bacteriophage was not logarithmic. The significance of this, and of similar and divergent results obtained by other workers with terrestrial bacteriophages, was discussed.

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Wanted, a Word. By S. T. COWAN (*National Collection of Type Cultures, London*)

Some scholars would have the greater include the lesser, but there are times and places where a new word to include both would be an advantage. An example is a single word for nomenclature and taxonomy to shorten (and improve) the cumbersome title *International Bulletin of Bacteriological Nomenclature and Taxonomy*.

First thoughts suggest a word based on the stems of both words, such as nomen+tax, but this Latin-Greek hybrid would make a classical scholar shudder. The Romans did not often combine words, but the Greeks did; therefore an appropriate word might be formed from two or more Greek words. The best I can think of would be based on *onoma*, name, and *taxis*, arrangement. From *onoma* the combining form is *onomato-*, and we have the word *onomatology*, the science of the formation of names, or terminology. To the stem ONOMATO-TAX- suffixes would make nouns and adjectives, such as ONOMATOTAXER, or ONOMATOTAXIST, one who deals with nomenclature (n) and taxonomy (t); ONOMATOTAXY, the subject of n and t; ONOMATOTAXOLOGY, the study of n and t; ONOMATOTAXOLOGIST, a student of n and t; ONOMATOTAXIC and ONOMATOTAXAL, adjectival forms.

These polysyllabic words are difficult to say quickly and I would welcome suggestions for a better stem. But, as a journal title '*International Bulletin of Bacterial Onomatotaxy*' is no worse than the present, and could with advantage be shortened to *Bacterial Onomatotaxy*, or *Bacterial Onomatotaxology*.

Production of Virulence in an Avirulent Strain of *Mycobacterium tuberculosis* by certain Non-ionic Surface-active Agents. By P. D'ARCY HART AND R. J. W. REES (*National Institute for Medical Research, London*)

When the avirulent strain of *Mycobacterium tuberculosis* H4Ra was repeatedly subcultivated in a liquid medium containing Triton A20 or the chemically similar, but simpler, non-ionic surface-active agent 'D4' (synthesized here), the morphology changed from the amorphous growth pattern and the circumscribed type of colony characteristic of avirulent tubercle bacilli to the forma-

tion of microscopic 'cords' and spreading colonies similar to those shown by virulent strain. These strains thus developed frequently produced a mortality when tested in mice; they differed from cord-forming H37Ra, produced similarly, whose virulence was as low as that of its unmodified parent strain. The produced virulence of H4Ra persisted after transfer to, and subculture in, medium devoid of surface-active agent. Washed suspensions of unmodified, amorphous-growing, H4Ra decolorized methylene blue rapidly; cord-forming, virulent strains produced therefrom by the above means showed delayed decoloration typical of virulent strains (low dehydrogenase activity). Cord-forming (but still avirulent) strains developed similarly from H37Ra retained the property of rapid decoloration possessed by the parent strain. The mechanism of the production of virulence in H4Ra, and the relationships of dehydrogenase activity, cord-formation and virulence in tubercle bacilli, are discussed. Dehydrogenase activity appears to be more closely related to virulence than is cord-formation.

Production *in vitro* of the Anthrax Toxin Previously Recognized *in vivo*.

By PATRICIA W. HARRIS-SMITH, H. SMITH and J. KEPPIE (*Microbiological Research Establishment, Porton, Wiltshire*)

Smith, Keppie & Stanley (1955) demonstrated the presence of a specific, lethal, oedema-producing, toxin in the plasma of guinea-pigs dying of anthrax. Until now, a specific toxin from *Bacillus anthracis* has not been obtained from artificial culture despite numerous attempts to do so. Knowledge of this toxin gained from *in vivo* studies has enabled us to produce the toxin in cultures grown in blood, plasma, or serum and we now know that its early appearance and rapid disappearance is the reason why it has not been recognized previously *in vitro*. Thus, if heparinized or defibrinated guinea-pig blood (30 ml.) is inoculated with 3×10^7 germinated spores of *B. anthracis* (N.P.) and shaken at 37°, toxin comparable to that in dying guinea-pigs was demonstrated at 4–5 hr. but none was found after 6–7 hr. Under similar conditions, toxin was produced and rapidly destroyed by the non-capsulated and hence avirulent 'Sterne' strain of *B. anthracis*.

The toxin formed an oedematous plaque when injected intradermally into guinea-pigs and given intravenously, it killed mice. The toxicity was of the same order as that of plasma from animals dying of anthrax and like the latter was specifically neutralized by anthrax antiserum.

Variation of the rate of shaking, aeration, proportion of CO₂, and temperature of incubation did not make marked differences in toxin production and as soon as the growth increased beyond a critical point destruction of toxin occurred.

Extracts of *Bacillus anthracis* from 7 hr. cultures rapidly destroy the toxin. It appears that this destroying mechanism is absent or limited when *B. anthracis* grows *in vivo* and to increase the yield of toxin *in vitro* a similar limitation must be achieved.

REFERENCE

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The Inhibition of Small Inocula on the Surface of Nutrient Agar. By
MARGARET M. TAYLOR (*Hannah Dairy Research Institute, Ayr*)

Small inocula of a thermophilic *Bacillus* are inhibited when plated on the surface of nutrient agar but not when growing within the medium. The inhibition is associated with Yeastrel and Lab-lemco especially when either of the two products is sterilized with agar. Neutralization is obtained with manganese dioxide, catalase, molybdic acid, nucleic acids, by sterilizing the broth and agar separately or by storing the inhibitory medium for 14–21 days. The inclusion of 1 % soluble starch gives partial relief in nutrient agar but has no further effect when the broth and agar are sterilized separately.

The inhibition can be reproduced by the addition of suitable dilutions of sodium sulphide or thioglycolate to non-inhibitory nutrient agar. This induced inhibition is reversed by manganese dioxide, catalase and the other compounds which were effective in the case of the inhibitory agar.

Comparison of the growth of *Shigella dysenteriae*, *Chromobacterium violaceum* and *Pasturella pestis* with the growth of the thermophile shows that these organisms are sensitive to the presence of Yeastrel or Lab-lemco. Neutralization is effected by the same compounds and partial relief is obtained by the addition of 1 % starch. While the thermophile grows better on stored media the other species are inhibited. These organisms grow on cellophane over inhibitory agar hence it is unlikely that the inhibition is due to hydrogen peroxide or hydrogen sulphide.

Experiments suggest that the inhibition is of the same type for all four micro-organisms and may be due to either an inhibitor present in small quantities which produces a marked sensitivity towards the oxidation-reduction potential of the medium or that the oxidation-reduction potential is inhibitory.

Two other species of thermophiles, *Bacillus brevis*, *B. subtilis* and *B. coagulans* also show this type of inhibition.

The Pathogenic Significance of Urease in *Corynebacterium renale*. By
A. J. LISTER (*Department of Biochemistry, University of Cambridge*)

The Chromatophores of *Chlorobium thiosulphatophilum*. By JANE GIBSON
(*A.R.C. Unit for Microbiology, Sheffield University*)

The photosynthetic pigments of plants are restricted to definite structures in the cell, the chloroplasts, which have themselves been shown to contain substructures, in which the pigment molecules are oriented. This situation has a parallel in the photosynthetic bacterium *Rhodospirillum rubrum*, in which Schachman, Pardee & Stanier (1952) have demonstrated the presence of particles which have a diameter of about 110 m μ . in electron micrographs, and with which both chlorophyll and carotenoid pigments are associated.

The green sulphur bacterium *Chlorobium thiosulphatophilum* has been examined to see whether particles of this kind are to be found in other photo-

synthetic bacteria. Cells have been broken down by freezing and thawing as well as by sonic oscillation, and a fraction corresponding to the *Rhodospirillum rubrum* chromatophores separated by differential centrifugation. Electron micrographs prepared from this fraction show the presence of round, flattened particles with a diameter of about 190 m μ . Unshadowed preparations show clearly also the presence of smaller particles, about 40–50 m μ , in diameter, and very dense to the electron beam; these are less obvious in shadowed preparations.

The Chlorobium particles differ from those of *Rhodospirillum rubrum* in that only a small part of the total pigment sediments with them. Differences in the absorption spectrum of the 'particle' and 'supernatant' fractions are apparent, but are not entirely consistent. In general, the carotenoid appears to sediment with the particles, which have a much less marked peak at 730 m μ , than either the whole cells or the 'supernatant' fraction. The differences observed between preparations may be due to alterations in the physiological state of the organisms used in making them. It may also be that the particles are more fragile than those from *R. rubrum*, and have disintegrated to a greater or lesser extent in all the preparations so far examined.

Thanks are due to Professor R. W. K. Honeycombe and Mr A. K. Seal of the Department of Metallurgy for permission to use the electron microscope and for help in the preparation of specimens.

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Some Properties of a Pectolytic Soil *Flavobacterium*. By M. J. DOREY (Department of Microbiology, University of Reading)

Soil samples from a number of different sites were perfused with pectin solutions and plated out on pectate gel, and organisms found to liquefy the gel were isolated for study. Many of these were Actinomycetes. In addition, a *Flavobacterium* sp. was constantly isolated; no other pectolytic bacteria were noted. The *Flavobacterium* sp. died out rapidly on nutrient agar, and it was found that this was partly due to the presence of an inhibitory substance which appeared to be a peroxide. The organism is unlike any *Flavobacterium* spp. so far examined.

The enzyme produced by this organism is an adaptive depolymerase activated by Ca⁺⁺. An agar plate test for this enzyme has been devised based on that for fungal polygalacturonase (Dingle, Reid & Solomons, 1953). The agar was washed, and Tris buffer at pH 8 employed. In order to demonstrate the requirement for Ca⁺⁺ silica gel must be employed in place of the agar.

REFERENCE

- DINGLE, J., REID, W. W. & SOLOMONS, G. L. (1953). *J. Sci. Fd Agric.* **4**, 149.

The following films were shown:

The Swarming of *Proteus vulgaris*. By W. H. HUGHES (*Wright-Fleming Institute of Microbiology, London*)

Flagella of Spirilla. By A. PIJPER (*University of Pretoria, South Africa*)

Spirillum volutans, *serpens*, *itersonii*, *cohnii* and *sinuosum* all show a helical body and a horny-looking tapering polar flagellum with a set curve, attached by a thin neck. They are all used in turn to illustrate aspects of motility. Motility is shown first with visible flagella, including to and fro movements when flagella appear passive. In the following scenes the body wriggles faster than the flagellum, or seems to move the flagellum, and motility also occurs without a flagellum showing. By contrast, *S. sinuosum* which was motionless is shown with numerous flagella. When a spirillum stops moving the flagellum is seen to wave and split, nothing to do with motility. True motility is demonstrated as alternating stretching and shortening of the coils of the helical body with intermittent free-wheeling. Autolysis of cell contents reveals the flagellum as a continuation of the cell-wall. Diagrams show how a cell-wall having a frame-work of fibrils can produce a terminal flagellum through cell division. Ageing cultures show additional variously shaped flagella all over the body. This is a result of excessive growth of these fibrils. Further ageing brings irregular outgrowths of fibrils looking like untidy darns of the cell-wall, these drop off as do the hardly recognizable flagella. The conclusion is that flagella are dispensable excrescences without motor function.

Members of the Departments of Botany and Zoology gave a series of exhibits and demonstrations of techniques applied to the study of Plant viruses (J. CALDWELL), Virus induced nuclear abnormalities in plants (J. WILKINSON), Fungi (G. C. AINSWORTH and T. J. WALLACE), Parasitic *limax*-Amoebae (R. J. S. HAWES and K. VICKERMAN), and Tissue culture of flowering plants (S. H. ROBB).

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